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Human ABO Blood Group and Anti-α-Gal Antibody Depletion in a Porcine-Human Working Heart Xenotransplantation Model of Hyperacute Rejection.

by

Rizwan Abdulmalik Samji Manji

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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A vision without a task is a dream.
A task without a vision is drudgery.
A vision with a task is the hope of the world.

– Unknown

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Human ABO Blood Group and α-Gal Antibody Depletion in a Porcine-Human Working Heart Xenotransplantation Model of Hyperacute Rejection" submitted by Rizwan Abdulmalik Samji Manji in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Experimental Surgery.

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To my Mom, my Dad and most importantly, my wife - Jacqueline Sarah Manji

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Parts of this work are in preparation for publication. The titles are as follows:

1. Methodological Considerations in the Development of a Hyperacute Rejection Porcine-to-Human Working Heart Xenotransplantation Model.
2. The Role of Human ABO Blood Group in Porcine-to-Human Cardiac Xenotransplantation.
3. Specific Depletion of Anti-α-Gal Antibody and Human ABO Blood Group in Porcine-to-Human Cardiac Xenotransplantation.

Abstract

Pig organs may solve the critical organ shortage crisis present today in allotransplantation; however, humans have naturally occurring antibodies against pig antigens (particularly the α-gal antigen) such that if human blood perfuses a pig organ, these antibodies bind to pig antigens leading to hyperacute rejection and failure of the graft. There are many similarities between the human ABO blood group antigens and the α-gal antigen suggesting that anti-ABO blood group antibodies may play a role in pig xenograft rejection. The purpose of this project was to determine if the human ABO blood group of a recipient effected survival of a porcine cardiac xenograft and whether specifically depleting human blood of antibodies recognizing α-gal antigen would prevent hyperacute rejection in a porcine cardiac xenograft.

A porcine-to-human working heart xenotransplantation model of hyperacute rejection was developed and validated. Various factors that were important in determining survival such as the anti-α-gal IgM and IgG levels as well as the presence of human blood group O red blood cells was elucidated as was the minimum amount of anti-α-gal antibody needed to cause rejection.

Having a valid model, *in vitro* and *ex-vivo* studies revealed that human blood groups A and O were most toxic to pig xenografts and blood groups AB and especially B were least toxic to pig xenografts. The mechanism suggested is that anti-B antibody (present in blood group A and O people) is toxic to pig xenografts and anti-A antibody (present in blood group B) is protective to pig xenografts.

Finally, it was determined that the immunoadsorbent S90/IB6 specifically depleted about 80-90% of anti-α-gal or anti-α-gal-like antibodies and not total antibody nor complement. This anti-α-gal depleted blood led to prolongation of porcine cardiac xenograft survival compared to a blank immunoadsorbent but the improvement was dependent on the human ABO blood group being tested. Even though there was significant depletion of the anti-α-gal antibody and improvements in survival, there was still significant human antibody staining on the pig hearts perfused with S90/IB6 treated blood suggesting that there are other antibodies present (besides anti-α-gal) in the pig-to-human combination which may be important.

There were many novel findings in this Ph.D. project with important clinical applications. These include choosing who may be best and worst to xenotransplant, who may be best and worst to immunoadsorb, pre-transplant parameters to measure (inexpensive, convenient anti-α-gal antibody level assays versus more difficult, expensive anti-pig antibody level assays) and pre-transplant therapy issues (O red blood cell transfusions).

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List of Abbreviations

180 fxn	Average 180 minute cardiac function
α1,3 GT	α1,3 galactosyltransferase
α-gal	Galactose α 1-3 Galactoseβ 1-4 N-acetylglucosamine Galactose α 1-3 Galactose β1-4 Glucose Galactose α1-3 Galactose
µg	Microgram
µl	Microlitre
µm	Micrometre
ABO	Human A, B, O blood group antigen system
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
bpm	Beats per minute
BSA	bovine serum albumin
C1-9	Complement components 1-9
C3a	Complement component 3a
C5a	Complement component 5a
C5b	Complement component 5b
C1q	Complement component 1q
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CD46	Membrane cofactor protein
CD55	Decay accelerating factor
CD59	Homologous restriction factor

CHF	Congestive heart failure
CK	Creatine kinase
cm	Centimetre
CO	Cardiac output
CO_2	Carbon dioxide
CORR	Canadian Organ Replacement Registry
CRP	Complement regulatory protein
CsA	Cyclosporine A
CVF	Cobra venom factor
D5W	5% dextrose in water
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DSPG	Deoxyspergualin
DXR	Delayed xenograft rejection
EC	Endothelial cells
ECI	Extracorporeal immunoadsorption
ELISA	Enzyme linked immunosorbent assay
ET	Eurotransplant
Fc	Crystalizable fragment (immunoglobulin non-antigenic binding fragment)
FITC	Fluorescein isothiocyanate
FK506	Tacrolimus
g	Gram
Gal	Galactose

GalNAc	Galactose n-acetyl
H & E	Hematoxylin and eosin
H epitope	α-1,2-fucosyl lactoasamine (O blood group antigen)
HAR	Hyperacute rejection
HBSS	Hanks' buffered salt solution
HCO_3^-	Bicarbonate ion
HCT	Hematocrit
hDAF	Human decay accelerating factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRF	Homologous restriction factor
HRP	Horseradish peroxidase
IB6	Immunosorb B6
IκBα	NF-κB inhibitor
IL-8	Interleukin 8
IM	Intramuscular
IV	Intravenous
ICAM	Intracellular adhesion molecule
IgA	Immunoglobulin alpha (class A)
IgE	Immunoglobulin epsilon (class E)
IgG	Immunoglobulin gamma (class G)
IgM	Immunoglobulin mu (class m)
IM	Intramuscular

IV	Intravenous
IVC	Inferior vena cava
K^+	Potassium ion
kg	Kilogram
KH	Krebs' Hanseleit
L	Litre
LPS	Lipopolysaccharide
M	Molar (mol/L)
Mabs	Monoclonal antibodies
MAC	Membrane attack complex
MCP	Membrane cofactor protein
mEq	Milliequivalent
mg	Milligram
MHC	Major histocompatibility complex
mL	Millilitre
MMD	Michelle's transport media
mmol	Millimol
mRNA	Messenger ribonucleic acid
MTT	3-4,5-dimethyldiazol-2-yl-2,5-diphenyl tetrazolium bromide
mV	Millivolt
n	Number of replications
NF-κB	Nuclear factor-kappa B
NHP	Nonhuman priamte

NK	Natural killer
nm	Nanometre
OCT	Tissue-tech OCT media
O.D.	Optical density
OKT3	Anti-CD3 monoclonal antibody
p	Probablility
PAEC	Porcine aortic endothelial cell
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDTC	Pyrrolidine dithiocarbamate
PEC	Porcine endothelial cell
PERV	Porcine endogenous retrovirus
pH	Logarithmic scale hydrogen ion concentration
RAPA	Rapamycin
RBC	Red blood cell
RCA	Regulators of complement activation
rpm	Revolutions per minute
sCR1	Soluble complement receptor type 1
S90	Synsorb 90
SEM	Standard error of the mean
SIV	Simian immunodeficiency virus
SVC	Superior vena cava

SWI	Stroke work index
TBS	Tris buffered saline
UKTSSA	United Kingdom Transplant Support Service Authority
UNOS	United Network of Organ Sharing
VAD	Ventricular assist device
Va	Coagulation Factor Va
VCAM	Vascular cell adhesion molecule
vWF	von Willebrand factor
Xabs	Xenoantibodies

CHAPTER 1

Background Information and Rationale

Chapter 1: Background Information and Rationale

I. Cardiac Allotransplantation and the Organ Shortage Crisis

With the important contributions of individuals like Carrel (1,2) - vascular anastomosis; Medawar & Burnett (3-5) - immunological tolerance; and Calne (6) – cyclosporine; the field of allotransplantation (transplantation within a species) has had considerable success. There have been over a million organ and tissue transplants performed world wide to date (7). In 1999, worldwide (~29 countries comprising ~790.5 million people), there were over 40,000 cadaveric solid organ transplants performed, with the most being performed in the USA – see **Table 1.1** (7).

The first heart transplant from human to human was performed on December 3rd, 1967 at the Groote Schurr Hospital in Capetown, South Africa by Dr. Christaan Barnard (8). This patient, like many others who followed him, survived less than a year after transplantation - most dying within a month usually of rejection or infection (9); however, survival rates for cardiac allotransplantation have been improving especially over the last 15 years (10). For cardiac transplantation today, the 1, 3, and 5 year survival rates are ~80%, ~75%, and ~65% respectively (11).

The mean age of heart transplant patients is about 50 years (12) and the main indications for heart transplantation are ischemic heart disease and cardiomyopathy (12). The number of heart transplants being performed has been decreasing over the last number of years (12). The main reason for this decrease is probably the success of preventative medicine (including the use of seatbelts and bicycle helmets, and automotive

safety issues), leading to fewer severe head traumas and therefore fewer organ donors (12).

Because of the fact that there are fewer organ donors, combined with a number of other issues (which will be discussed shortly); we are currently facing a critical organ shortage. **Table 1.2** shows the number of patients waiting for various organs compared to the number transplanted the year before in the USA (12). The overall average shows that only ~30% of people who need an organ are transplanted. It is estimated, that by the year 2010, the number of people on the waiting list will have increased by about 200% (i.e. ~225,000 people); whereas the number of organ donors will likely stay static around the 20,000 mark (in the USA) if nothing is done (13). This means that instead of the current 30% of people who get transplanted, only about 10% of people on the waiting list will get transplanted.

The current shortage is so severe partly due to inability to use potential donors. Studies of hospital indexes (14,15) have suggested that around 14% of those dying in the intensive care unit and 2-3% of all people dying in hospital will suffer brain death. However, 17-20% of these will have medical contraindications to organ donation. Others will have a complication while waiting to have organs retrieved. In a hospital in Barcelona, over a 5 year period, 14% (55/399) of otherwise acceptable organ donors suffered from either a cardiac arrest or uncontrolled sepsis making them unsuitable to be organ donors. Another percentage of potential donors will not be used because of family opposition or other problems (16). Studies done in Spain, France, and Madrid showed the overall refusal rate of a donor ranged between 16% to 25% (16). Even if the donor makes it to the operating room, there may be damage to the organs making their use

suboptimal or not possible (16). A report to the UKTSSA Kidney Advisory Group in 1997 (16) showed that ~20% of kidneys were being damaged during retrieval. Most were reparable; however 1% of all organs (kidneys, hearts, lungs, livers) retrieved could not be used.

Some other reasons that the organ shortage crisis is expected to get worse includes the fact that the "baby boomers" are in the age range where they may need an organ transplant secondary to ischemic heart disease or other pathology increasing the demand for organs (13). Also, as transplantation generally works very well, the indications for transplantation are increasing (13). Thus, there currently is a severe organ shortage that is only expected to get worse. In the next section we look at possible ways to address this shortage.

II. Sources of Organs and Alternatives to Allotransplantation

Many methods are either in place or are being explored to increase the organ donor pool. These methods can be divided into regularly practiced, newer methods in clinical practice, methods still in the laboratory, and controversial methods.

Regularly practiced methods include making the general public more aware of the organ shortage problem as well as of the importance and success of allotransplantation. The medical literature however, shows that increasing transplantation awareness of the general population may overall be ineffective (16). A television campaign conducted by the Department of Health in the United Kingdom showed a drop in the refusal rates from 30% to 22% during a period of intense publicity that soon returned to pre-campaign levels. Feelings are that campaigns are ineffective or at least have a very high cost-effectiveness ratio.

Medical professionals need to keep transplantation in the back of their minds with respect to any patient they see who may become brain dead. Many doctors are now even accepting organs and tissues of older patients, who normally would not have been considered donors, as well as younger patients who may have needed more than desired hemodynamic support as organ donors (16). Because many health care practitioners may not think of or seek consent of families for organ donation and because some families may not want to give consent, certain countries have instituted more aggressive regulations for organ donation (16). These countries have "presumed consent" which means that organs are harvested in every case unless one specifically refuses. Countries that have presumed consent include: Finland, Portugal, Austria, Sweden, Czech Republic,

Slovak Republic, Hungary, and Poland. Countries that theoretically have presumed consent but practically have informed consent include: Spain, Italy, Greece, Belgium, and France. Countries that function strictly on informed consent include: the United States of America, Latin-America, the United Kingdom, Ireland, Denmark, the Netherlands, Germany, and Canada. **Table1.3** (16) shows the cadaveric organ donor information for 1999 categorized by method of consent. Looking at the table reveals that presumed consent does not increase the number of available organ donors.

In many places like Japan, organ harvesting from brain dead individuals has been culturally unacceptable until just recently (17). In these places living organ donors are used quite successfully (18). This method is also common in other parts of the world like Canada and the United States, especially for kidney transplants (19). More recently, living-donor liver transplants have become more popular (20,21). The use of living people as organ donors works, however, there are risks to the donor. As successful as living donor transplantation is, it still will not meet the needs of all those that need organs, especially those who need organs such as the heart.

Other methods of increasing the donor pool of human organs includes using non-heart beating donors. In many countries, for cadaveric allotransplantation, organs are harvested from an individual who is brain dead but whose other organs (such as the heart) continue to function with hemodynamic and ventilatory support. In places like the Japan (22-25) and also the United States (26-28), surgeons are performing transplants with non-heart beating donors. This method is used for many organs such as the kidneys, and liver; however, organs such as the heart cannot be used. Results are encouraging but

again, this source will not meet the large demand existing at present and it does not work for organs like the heart (23,28).

Some of the lab research work to increase donor availability includes ways to improve donor organ preservation/storage and recovery so that organs can be stored for longer periods of time to allow them to be transplanted in the best possible patient for that organ even if that patient is a very long distance away (29-32). Research is being done but a solution that can be brought into standard clinical practice does not yet exist.

There is also very exciting work in genetics and cloning which may help with the organ shortage crisis. Researches are now able to clone animals (sheep, pigs) from an adult cell and are also able to grow tissues such as skin and islets in the laboratory (33). It may be possible to combine all this knowledge and these techniques to clone human organs from the patient's own organ cells. This would be a major breakthrough in transplantation and may actually be the best solution to the organ shortage crisis. However, at present, it is not possible to clone and grow specific organs and this technology will likely take some time (if at all) to develop and perfect. Another reason why organ cloning is not a feasible option is the fact that if a patient needs an organ immediately because of acute heart failure or acute liver failure, there likely will not be enough time or enough healthy patient cells to be able to grow an individualized organ in time to transplant into the patient. Organ cloning may prove to be best for chronic organ failure (e.g. cystic fibrosis, chronic heart failure) as opposed to acute organ failure.

Controversial sources of organs and tissues include using organs and tissues from executed prisoners, aborted or anencephalic fetuses and paying people to donate sparable (or perhaps unsparable) organs. These have significant ethical and practical concerns.

All of the above methods of increasing the donor pool are not expected to be able to meet the demand for organs at present and in the near future. However, xenotransplantation may be able to meet these needs (as discussed later).

Many have looked at other methods of addressing organ failure besides transplantation. For heart failure, surgeons have long used mechanical devices such as ventricular assist devices and total artificial hearts (34,35). These have the advantages of avoiding rejection; however, there are problems including intracranial bleeding if anticoagulation is too high causing head trauma, device malfunction (mechanical or thrombotic), infection, expense, the inability to use the device in patients with a body surface area less than 1.5 m^2 and inconvenience due to the connection of the patient to a console (35-37). Currently, mechanical devices are usually used as a bridge to either allow the patient's own heart to recover function or to allow them to function until a suitable human donor becomes available (38). However, there is work taking place in mechanical therapies with the intent of having mechanical devices used instead of transplantation (39,40). There is still work to do, but permanently supported mechanical hearts may rival xenotransplantation as an ideal means of dealing with heart failure. However, problems would still probably include inability to quickly adjust to physiological demands (41), and mechanical problems. Even if mechanical therapy proves superior to xenotransplantation for heart failure; there are many other organs/ tissues which are difficult or impossible to replace with mechanical therapy and thus xenotransplantation may be best for these.

Other methods to deal with heart failure have included cardiomyoplasty and the Batista procedure. Cardiomyoplasty is the use of skeletal muscle power to augment

ventricular function. Since it was first clinically done by Carpentier (42) in 1985, there have been at least 2 multicenter prospective trials on cardiomyoplasty (43,44) The first study (1985-1991) reported a 21% mortality, which had decreased to 12% by the second trial (1991-1993). However, this survival rate was the same as for a prospectively followed control group that was matched for the degree of heart failure. In the final analysis of the second trial, it was found that one year survival following cardiomyopasty was only 68% which is the same as the survival of congestive heart failure (CHF) patients without surgical intervention. The current application of this technique is solely for the treatment of CHF; however, a better treatment for CHF would be heart transplantation. Batista removed part of the ventricular wall with the intent of trying to normalize anatomy which may normalize function (Batista procedure) (45-47). His method is used by some but is not widely performed as there are concerns about removing parts of the heart.

Newer research in the area of treating heart failure deals with stem cell research. Stem cells are undifferentiated cells which have the potential to develop into any type of cell. People who have had multiple heart attacks have many areas of their heart muscle replaced with scar tissue. Progressive cardiac dysfunction results and this leads to heart failure, end stage heart disease and the need for transplantation. Researchers are looking at the potential of using stem cells to help regenerate myocardium and thus prevent heart failure (48-52) Though this is in its early stages, this form of therapy may have a significant impact especially in people who are poor surgical risks. This form of therapy again would likely be more for chronic organ failure as opposed to acute organ failure.

All of the above are possible sources of organs and possible alternatives to organ transplantation. Each have advantages and disadvantatges. Xenotransplantation (transplantation between different species) is another possible source of organs and tissues for human transplantation that may solve the organ shortage crisis and may be possible to achieve in the near future. Xenotransplantation will be discussed next.

III. Xenotransplantation

A) Definitions

Allotransplantation is the transplantation of organs and tissues within a species. Xenotransplantation, on the other hand, is the transplantation of organs and tissues between different species (10). There are two types of xenografts, concordant and discordant (53). Concordant xenografts are grafts between phylogenically close species such as mouse to rat or chimpanzee to man. These combinations do not undergo hyperacute rejection but undergo a less severe form of rejection termed acute vascular rejection/delayed xenograft rejection (54). This form of rejection develops within a few days of transplantation and induced antibody (usually IgG) is important in its development. Discordant grafts on the other hand are between phylogenically disparate combinations (such as pig to human), where one has naturally occurring antibodies, usually of the IgM isotype, against the other. These combinations undergo a very rapid (minutes to hours) form of rejection termed hyperacute rejection (discussed in section on hyperacute rejection) (54). A discordant species (pig) has been chosen as the most likely organ donor (details of which will be discussed below).

B) History of Xenotransplantation

Ideas of xenotransplantation date back centuries as can be seen in stories of mythological beings that were more powerful since they had the advantages of being part animal as well as part human (13). The first surgical attempt at xenotransplantation was reported in 1804 when Boronio described the failure of a cow and horse skin graft

exchange (55). Similar failures were reported by Bert (55) who tried to transplant guinea pig skin to rats. In the early 1920s, Russian surgeon SergeVoronoff transplanted 51 older human males with baboon testicles in an attempt to "rejuvenate their physical, mental, and sexual condition" (13). Voronoff writes of how successful his procedure was; however, with today's knowledge, we know that these grafts probably did not survive very long and most of the beneficial effects were probably placebo effects.

i) Solid Organ Xenotransplants

The first vascularized renal xenotransplant in a human was described by Jaboulay in 1905 (55). Jaboulay transplanted two patients - one was pig to human and the second was goat to human. Both of these grafts failed and pathology showed vessel thrombosis. There were a few other failed trials early in the 20th century and then the interest in xenotransplantation subsided. Interest picked up again in the 1960's when Reemtsma (56) did xenotransplantation using immunosuppressive protocols that were known to blunt allograft rejection. Reemtsma did 12 chimpanzee to human renal transplants from 1963-1964 using an immunosuppressive protocol of steroids, actinomycin C and local irradiation of the graft. Most of the xenografts rejected within 2 months; however, one functioned for greater than 9 months and at the patient's death (from infection), there was little evidence of rejection in the graft. Starzl *et al.* (57) and Milliard *et al.* (55) did 6 baboon to human kidney transplants in the early 1960's as well. Even though they used similar immunosuppression as Reemstma had used, the grafts functioned only a mean of 36 days (range 10-60 days). In their studies there was no evidence of hyperacute rejection but there were more frequent and more severe rejection episodes. Pathology showed hemorrhagic infarcts compatible with vascular

lesions characteristic of humoral rejection (57). The baboon is more phylogenetically disparate (further away) from humans than are chimpanzees and as described in more detail later, the more phylogenetically disparate the combination, the more humoral the rejection response. This explains (at least to an extent) why Starzl and Millard had shorter survivals with their transplants as compared to Reemstma (even though the immunosuppression regimen was similar).

In 1964, Hardy *et al.* (11) performed the first heart xenotransplant (using a chimpanzee donor); however, the recipient died on the operating room table and the cause of death was said to be an undersized graft and perhaps hyperacute rejection contributing to cardiac failure. In 1984, Bailey (58) transplanted a baboon heart into an infant (Baby Fae) with hypoplastic left heart syndrome under cyclosporine immunosuppression. The graft functioned well for about 2 weeks with subsequent problems leading to cardiac failure and death at 20 days postoperatively. The pathology was very educational. There was only minimal cellular infiltrate (suggesting that cyclosporine had been effective); however, there was evidence of interstitial hemorrhage and coagulative necrosis implying that humoral rejection was predominant and this latter finding was confirmed by demonstrating antibody and complement deposition on the vasculature (58).

Realizing the importance of the humoral arm of rejection in xenotransplantation, Starlz's group preformed two liver transplants from baboons to humans between June 1992 and January 1993 (11). This was done under immunosuppressive coverage to target both the humoral and cellular arms of the rejection process with tacrolimus, steroids, cyclophosphamide and prostaglandin E1. One recipient died at 25 days (of

infection-peritonitis), and the other died at day 70 (of infection). Pathology did not show any humoral or cellular rejection in either case, but there was evidence of complement activation. Starzl concluded that xenotransplantation may be achievable once complement activation is overcome.

The next important xenotransplant performed was in 1993 by Makowka (59). A porcine liver was used as a bridge to support a young woman with hepatic encephalopathy until a liver allograft became available. The graft did function for a period of time, but the patient deteriorated, being declared brain dead prior to getting allo-transplanted. Makowka deduced that the graft failed because of a rapid return of xenoreactive natural antibodies against the graft following transfusion of the patient with human blood products. This deduction was indirectly confirmed by pathology consistent with hyperacute rejection (60). Thus, from 1906 to 1992, there have been over 40 attempted human xenotransplants and all have failed; however, all have provided useful information about the xenorejection process such as the importance of phylogenetic distance and the importance of anti-body mediated rejection (**Tables 1.4-1.6**).

ii) Extracorporeal Xenograft Perfusions:

Because of problems with solid organ xenotransplantation, some have investigated extracorporeal perfusions. Extracorporeal perfusions are done outside the body and involve passing the recipients blood through the donor organ or through a system of types which has the donors cells lining them. An advantage of extracorporeal perfusions is technical. Since the xenograft is outside the body once it rejects another xenograft can more easily be attached versus having to do a more involved operation to harvest the transplanted organ from within the recipient. **Table 1.7** shows the

extracorporeal xenoperfusions that have been done (61). Most perfusions have been done for liver failure. The results are encouraging so far. Currently, researchers are looking into further development of extracorporeal xenograft perfusions (13).

iii) Cellular Xenotransplants

There have also been a number of cellular xenotransplants performed (particularly in the last half of the 1990's). **Table 1.8** lists some of the clinical trials conducted with various xenogenic cells (61). Advantages of the use of xenogenic cellular transplants as opposed to vascularized whole organ transplants are many. Firstly, as cellular xenotransplants are not immediately vascularized, they do not undergo hyperacute rejection though they can still undergo acute vascular rejection (11,62,63). Secondly, cells can be more easily manipulated compared to organs. Cells can be encapsulated (64,65) or treated to remove certain epitopes, genetically engineered (11,66) to express certain factors (e.g. human complement regulatory proteins or anti-apoptotic factors), or injected into "immunopriviledged sites" like the brain. These factors help to decrease the likelihood of rejection of xenogenic cells as compared to solid organs (11,13). Thirdly, cellular transplants are technically easier and physically less demanding on the patients than a major organ operation. The last theoretical advantage of cellular transplants is that they are less likely (than large and solid organs) to be an infection hazard because the number of cells (and thus potential infectious agents) transplanted is less than would be transplanted if a solid organ were used (33). This latter reason is why the FDA has allowed cellular xenotransplant clinical studies to continue while banning any further solid organ xenotransplant studies (33).

Looking at **Table 1.8**, the clinical results from cellular transplantation are not overwhelmingly encouraging nor discouraging but they suggest that further studies are needed. A reason that some of the results are not as encouraging as hoped may simply be a methodological issue (such as incorrect number of cells injected, improper processing of cells for implantation, need for xenogenic growth factor injection in addition to cell transplantation, need for larger sample sizes) (61). Hopefully, as further studies in cellular xenotransplantation continue, the results will be more positive.

C) Advantges of Xenotransplantation

Xenotransplantion has many potential advantages. There are approximately 90 million pigs slaughtered each year in the United States alone for food (13). If each one of those pigs' organs were used for transplantation, we would have a surplus of organs (at least in the United States) where currently 75,000 patients in total are on the waiting list for an organ (12). As there would be a surplus, much more flexible rules could be applied in choosing recipients (e.g. elderly patients, patients with cancer, patients with multisystem organ failure). These patients, with a transplant, may be able to overcome the disease process that spiraled the series of events leading them to their current situation (e.g. multisystem organ failure). In addition, with a surplus, the indications for transplantation may increase such that certain conditions, which may not be best managed medically, may be treated quite well with a transplant leading to a better quality of life. For example, type I diabetic patients are often young and may have poor glycemic control with serious complications such as hypoglycemic coma, diabetic ketoacidosis, and other chronic problems (vasculopathy, nephropathy). Type I diabetes mellitus can be managed medically but may not be managed optimally by medical therapy alone. For

their active lifestyle, frequent monitoring and adjusting of their blood sugars may be a nuisance leading to poor quality of life and some of the complications mentioned above. Recent studies have shown that transplanting the islets of 2-4 donor pancreata can lead to patients having excellent glycemic control with no need for any exogenous insulin (67,68). A significant problem is not having enough donor pancreata for the number of patients that could benefit from an islet transplant. With xenotransplantation, and the organ surplus, porcine islet transplantation may be first line therapy in those patients (33). Some scientists feel that many ailments known to man could potentially be cured by xenotransplantation (**Figure 1.1**).

Another advantage of xenotransplantation is that there likely would be much less organ injury than that which occurs with allotransplantation (13). Many feel that the injury of harvest, preservation, ischemia, and reperfusion contribute significantly to endothelial cell dysfunction which contributes to organ rejection (11,13,69). Some feel that this non-immune injury is much more important than immune mediated injury in eventual graft failure (70). With readily available organs, surgeons could plan transplants more precisely, at a time of day when there are enough support staff and everyone involved is more alert and situations are more controlled. The ischemia time of the organ donor could be much less as the surgeon transplanting the recipient could let the donor harvest team know when to start. All of these would lead to a "healthy organ" being transplanted.

A third advantage of xenotransplantation is that donor animals could also be genetically modified (to eliminate or express certain factors) to prevent organ rejection. Such genetic modification of organs is of course much harder in human donors. Genetic

engineering has already been attempted and tested (71,72) and this will be discussed later; however some points are presented here. One of the main reasons that human organs are rejected is because we express human leucocyte antigens (HLA) on our tissues. These antigens identify our tissues as distinct from another's tissues. Unfortunately, this "identity marker" lets a recipient's immune system know that the donor tissue is foreign (not self) and thus the immune system attacks the donor tissue. If it was possible to eliminate the immune system's recognition of the donor foreign tissue (by removing all HLA for example), this may prevent rejection. Genetic engineering of animal organs may allow one to eliminate or down regulate antigens, possibly preventing rejection. If this is not possible, it may be possible, using transgene technology, to create organs which can inhibit the immune system by expressing factors (e.g. cytokines) that inhibit immune activation. Some of this has already been done with pig organs (hDAF pigs will be discussed later). Lastly, with genetic engineering, "super organs" may be created. We know that molecular biology techniques are used to make agriculture better (73). We may be able use similar technology to create hearts which are less likely to develop diseases like atherosclerosis. The advancements with the human genome project and the future identification of disease causing genes will undoubtedly be beneficial in genetically engineering "super organs".

Another benefit of xenotransplantation is that certain societies who have some cultural difficulties harvesting organs from a brain dead humans – such as the Japanese (17) - would probably no longer have this concern if animal organs were used. If this is so, then many people in countries like Japan could benefit substantially. Thus there are a large number of advantages to xenotransplantation.

D) Disadvantages of Xenotransplantation

There are a number of issues that one needs to be aware of concerning xenotransplantation. The first issue deals with ethics of using animals for human transplantation. Animal usage by humans has generally been accepted as long as the animals are cared for in a humane fashion (13). Livestock are currently bred and raised for consumption. Animal parts (porcine heart valves, porcine and bovine pericardium) are already used in humans today. Because of infection concerns (see below) and because of a potential "xeno organ donor market" that may develop, breeders of pigs for organ transplantation are likely to care for their animals quite well to ensure that they can stay competitive in the market by having good products. Thus, ethical concerns about using animal organs for human usage will probably just focus on ensuring that the animals are cared for and that organs are harvested in a humane manner not causing the animal undue stress or pain.

Another potential disadvantage of xenotransplantation is the possibility of infection transfer from animal organ to the human recipient and then possibly to contacts of the recipient. Researchers (74,75) have found that a porcine cell line having the ubiquitous porcine endogenous retrovirus (PERV) can infect human cell lines in the lab under certain simulated conditions. This is a concern; however, there have been no consequences documented from this infection. Researchers have also tested the blood of patients exposed to pig xenografts via PCR and have found no evidence of PERV infection (76). Many researchers feel that xenogenic infection (xenosis) will probably not be a major problem for reasons which include the following: firstly, infection spread is usually species-specific and as a result it is less likely that pig infectious agents will

easily infect humans and cause problems; secondly, facilities used for breeding pigs will be highly regulated and controlled and only "clean" pigs will be bred thus making baseline infection in the pigs less likely; in fact, inbreeding of transgenic pigs in Boston has shown that some of the pigs have less or no PERV in their genetic makeup; and thirdly, humans have used pig products for many years (pig heart valves, porcine insulin, pig meat) without serious problems thus if there was something very serious, it probably would have manifested by now (33,76,77). Even if PERV does lead to infection in a human recipient, it is hypothesized that the problem caused will likely be a lymphoma-like illness which has a probable chance of being cured by current protocols (33,78). Because all infection issues are still not well known, regulatory agencies have been formed to make some basic rules that xeno-researchers would have to comply with before trying human clinical trials (33). Also, organized bodies have formed to educate and poll the public on whether they think xenotransplantation should go ahead. Some of these reports are due to be completed sometime during this year (33).

Other concerns with regards to xenotransplantation deal with economics and regulation. It is likely that pig organs are going to (at least initially) be very expensive as researchers try to recover their costs related to their research and regulated breeding of pigs etc. The question is, who should pay for these organs - society (who potentially may be at risk from infection) or the individual (who may not be able to afford it). In addition, there will need to be regulation to ensure that procedures are followed that are in the patient's and society's best interest as opposed to just business's best interest. The question is who will regulate it and how will it be enforced? (11,13,33)

Other problems with xenotransplantation deal with perception issues. Will people feel not totally human if they have an animal's organ? Will xenotransplant recipients be ostracized from society because they are now "part animal"? These issues will have to be kept in mind as clinical trials of xenotransplantation are approached. Another perception issue that will need to be kept in mind is the fact that if people feel that xenotransplantation will solve the organ shortage crisis, they may decide that there is no longer a need for human donors which could be a greater problem if xenotransplantation is not successful.

There are significant advantages and disadvantages to xenotransplantation. Society will ultimately have to decide whether xenotransplantation should be attempted.

E) Choice of Organ Donor

There are a number of different animal species that have been looked at for human transplantation (11). These include birds, reptiles, lower mammals (e.g. pigs, sheep, cow) and higher mammals (e.g. apes, and monkeys).

Animal species that are evolutionarily closer together would likely be best to transplant as antigen similarity between the two would hopefully avoid very severe rejection. If one looks at the dissimilarity index (**Table 1.9**) the best animals to choose are the great apes which include chimpanzees and gorillas (11). Many of the early xenotransplants done in humans actually used organs from the great apes (such as the chimpanzee heart that was transplanted into Baby Fae). These nonhuman primate (NHP) to human organs did not undergo hyperacute rejection but underwent a less severe form of antibody mediated rejection called acute vascular rejection (11).

Problems with the use of great apes, and in fact any NHP, are many. The first is ethical considerations. As NHP are quite close evolutionarily to humans, there is a moral dilemma as to whether it is right to breed a higher animal species specifically for the purpose of killing them for human transplantation. Another major concern with the use of NHP is infection transmission. In general, the closer two animal species are to each other, the more likely that an infectious organism in one will infect the other and the more likely that this infection will cause problems (33). It is known that human immunodeficiency virus (HIV) comes from transmission of simian immunodeficiency virus (SIV) from NHP (77). Also, herpes B which can be present in NHP can very easily be transmitted to humans (eg. splash to eye) and is usually fatal within days (77). The high probability of infection spread and infectious related complications with use of NHP is likely enough to totally eliminate them as potential organ donors (79-81). Even if infection could be controlled, NHP still would not be a good source of donors because of size considerations (10). Some NHP (eg. chimpanzees) would never have organs large enough to support an adult human. Indeed, the first ever human heart transplant was done in 1964 by Dr. James Hardy using a chimpanzee heart. The patient died on the operating table because the chimpanzee heart was too small to support the circulation of the adult patient (13). The other reason that great apes are not suitable organ donors is there are not enough great apes to meet the demands of transplantation at present. There are enough old world monkeys, such as baboons, that could meet the current demand for organs, however, there would soon be a shortage again due to the fact that NHP are more difficult to breed to large numbers than other lower mammals (13,55). NHP often only have 1-2 offspring per pregnancy, gestation lasts 173-193 days, it takes 3-5 years before the offspring are

able to mate and it takes 9 years before the NHP would be large enough to transplant into a child (**Table 1.10**) (13). All of these factors related to breeding NHP make them not well suited for genetic engineering and the breeding factors plus measures for infection control would make NHP organs very expensive.

Other species that may be used for xenotransplantation are some of the lower mammals. Pigs have been looked at very favorably for many reasons. Ethical concerns are less volatile with pigs since they are slaughtered (over 90 million in the United States each year) for food on a daily basis (13). Though there is a risk of infection transmission to humans with pigs, this is less of a concern than with NHP. Anatomically, pig organs should work. The domestic pig would not be suitable as they are usually very large (>200kg) and thus their organs (especially heart) would likely be too big to transplant into a human. However, there are many strains of miniature pigs that have been bred that weigh about 70-80 kg when fully grown and thus would have organs of suitable size for human transplantation. In addition, pigs have a natural lifespan (if not slaughtered for food) of about 12-15 years and they usually reach adult size by about 6 months of age. Thus transplanting a pig organ after the pig has reached adult size should work for at least 10 years (just considering lifespan issues of the organ). Pig organs (such as kidneys and heart) have been transplanted into baboons and have been able to support the baboons for significant periods of time before rejection caused organ failure (11,13,82,83). Having an unlimited supply of pig organs and tissues should be obtainable almost immediately because of the breeding characteristics of pigs (**Table 1.10**). Sows have large litters (5-12 per litter on average), they have short gestational periods (114 days) have frequent gestational periods, have frequent estrous cycles, reach sexual maturity by ~4-8 months

of age and reach adult/transplantable size by about 6 months of age (13). Because of these breeding characteristics, genetic engineering would also be much easier in pigs, and also, the cost to have pig farms infection free for transplantation would be much less expensive than for NHP and thus the organs for transplantation would be cheaper than organs from NHP (13). There are some disadvantages to the use of pig organs. The DNA disparity between pigs and humans is large confirming the large phylogenetic distance between the two species making the pig-human combination a discordant combination (55,84). Because of this, humans have naturally occurring antibodies to pig antigens (particularly the α-gal antigen) and thus humans hyperacutely reject pig organs (11,85). Because of this problem with hyperacute rejection, investigators have looked for low α-gal expressing pigs; but unfortunately there is no specific strain of pig that are low α-gal expressers. Pigs express the α-gal antigen in a normal distribution with the high expressers having 5-7 times more α-gal than the low expressers (86). There are no pig strains that exist that do not express α-gal at all (87). Because of the problems with the α-gal antigen, investigators have looked at other species as potential organ donors. The ostrich is a possible donor because of anatomical and physiological reasons and non-mammals such as birds and reptiles do not express the α-gal antigen (88). Unfortunately, experimental studies demonstrated that these species express other antigens (such as β-gal) to which humans have naturally occurring antibodies leading to rejection (88). For now, pigs are the animals that show most promise for xenotransplantation.

There are other potential problems with the use of pig organs for xenotransplantation. Humans are a species that stands upright and are generally active. The human heart is designed for an active lifestyle and to function for an upright posture

(there are different pressure loads put upon the heart depending on posture). The pig has a horizontal posture and most pigs are quite sedentary. For all of these reasons, there are concerns that pig hearts will not be able to work in humans (89-91). Pigs which will be used for transplantation will likely have to have a very refined diet and may need to be exercised fairly aggressively and regularly (13). The above maneuvers will still not solve the posture issues. For this reason some investigators have been looking at the kangaroo as a potential donor (13). This may prove to be an important source; however for the time being because of expense, number, breeding issues, and other factors, the pig is still felt to be the animal to concentrate on.

There are other major concerns related to the differences in physiology between pigs and humans (11,89,90). **Table 1.11** lists some of the electrolyte differences between pigs and humans (13). Some of these differences are subtle but may become important. Other differences include that normal body temperature for a pig is 39°C whereas it is 37.4°C for humans. It is known that enzymes are very sensitive to temperature changes. Whether enzymes in pig organs will work as efficiently in a human body is not known. Certain hormones (such as growth hormone) are very species specific and it is unlikely that pig organs will respond properly to some of these hormones (13). It is possible that humans may need to be injected with certain pig hormones for the pig organs to respond appropriately. This may be problematic if humans subsequently produce antibodies to these injected proteins. This hopefully will not be a problem as the humans will likely be quite heavily immunosuppressed. If there are problems, human recombinant proteins may need to be engineered which can still deliver the appropriate signals to the pig organs but that are less immunogenic. If all of the above end up being problems, then transplants

may only be done of organs and tissues that are not as sensitive to hormones or that do not need much hormone input (e.g. adult pig heart into and adult human versus a neonatal pig heart which needs to grow in a neonate).

Just as pig organs may not respond to human proteins (such as hormones or growth factors), the proteins made by certain pig organs (especially the liver) may not function appropriately in a human. The liver is a very important biosynthetic machine making among others, coagulation proteins, albumin (to which many other substances attach for transport purposes), and complement proteins. Studies (13) have shown that porcine albumin is quite different from human albumin (**Table 1.9**) and this may cause problems with other substances being able to bind to the albumin for transport. Another problem may be pig complement. One of the ways that an organism protects itself against its own complement proteins is via complement regulatory proteins (CRP) which are present on endothelium. Indeed, researchers have used the species-specific knowledge about CRP to successfully make transgenic pigs which express human CRP and do not undergo hyperacute rejection (92). Pig livers will produce pig complement which may attack normal human tissue as normal human tissue will not express pig CRP (89).

Some of the issues with the use of pig livers may be addressed by the following. One option is not to use pig livers for xenotransplantation and just xenotransplant other organs which are not major biosynthetic machines (e.g. hearts, kidneys). This option may be difficult to accomplish as in addition to the problems already mentioned with the use of pig livers, the livers may be a constant source of pig antigen stimulation to the human immune system due to the constant production of pig proteins (11). Another solution may

be to make humanized pig livers through molecular biological techniques so that human proteins will be made by the liver. This option may be impractical. The other option (which may be the best overall plan) would be to use pig livers or isolated pig hepatocytes in a bioartificial system as a detoxifying agent only. This technology has already been used successfully many times in patients with fulminent hepatic failure to either allow time for the patients' own liver to recover or to bridge the patient to human liver allotransplantation (33,61).

Researchers have not concerned themselves with trying mammals such as cows, sheep, or goats due to size and availability concerns (93). In addition, all of these animals will likely have similar problems (in terms of physiology, xenoantigen production by the liver, etc.) as the pig. Though there are some concerns, currently, the pig is still felt to be the most suitable organ and tissue donor.

IV. Xenograft Rejection

Discordant solid organ xenografts undergo a very severe form of rejection referred to as hyperacute rejection (HAR) (94-96). If HAR is overcome, discordant grafts undergo acute vascular rejection and/or delayed xenograft rejection (11,85). There is also acute cellular rejection of xenografts; however, only a limited amount of information is known about this in a discordant combination at this time. The primary mode of rejection in the discordant combination is humoral (antibody-mediated) rejection and the primary mode of rejection studied in this project is hyperacute rejection. For these reasons, hyperacute rejection will be discussed in detail and acute vascular rejection will be discussed briefly.

A) Hyperacute Rejection

Hyperacute rejection (HAR) occurs in minutes to hours of anastomosis of a vascularized solid organ in discordant xenografting. There is binding of natural antibody (mostly IgM, but also some IgG) to endothelial cell antigens and this leads to activation of the complement cascade which leads to endothelial cell (EC) activation and may lead to EC death (11,97-99). Activation of the EC causes it to undergo numerous changes, which leads to cell injury as well as thrombus formation in the graft vasculature causing graft ischemia and ultimate organ failure (100,101). Pathologic examination of tissues having undergone HAR show deposition of antibody and complement on the graft vasculature as well as evidence of interstitial hemorrhage, and intravascular coagulation (102,103). *In vitro* studies using porcine endothelial cells (PEC) show that human IgM kills PEC mostly by binding complement; however, *in vitro*, human IgG kills PEC by antibody dependant cell cytotoxicity (104) (**Figure 1.2**).

Before further progression, a few things need to be expanded upon. These include identified natural antibodies (or xenoantibodies – Xabs) of interest in a pig to human discordant combination, complement, and EC activation.

i) Xenoantibodies of Interest in the Pig-to-Human Combination

There are a number of porcine endothelial cell (PEC) (105) glycoproteins recognized by human Xabs. All of them have not been fully characterized, but it is known that they are members of the integrin family (105). The primary entity on PEC that is recognized by human Xabs and that is the cause of hyperacute rejection is the Gal α 1-3 Gal (α Gal) epitope (98,99,106-111). α-gal is the short form denoting the following sugars: (i) gal α1,3 gal, (ii) gal α1,3 gal β1,4 Glc and (iii) gal α1,3 gal β1,4 GlcNAC.

The anti-α-gal antibody was first discovered around 1983 by Uri Galili who was studying mechanisms of destruction of abnormal human red blood cells (RBC) (sickle cell, β-thalassemia) (112,113). As was learned later, abnormal red blood cells and old senescent red cells are bound by anti-α-gal antibody and can be removed in this way (112-116). When Galili was first doing his studies, he did hemagglutination inhibition experiments using various sugars and found that very specific sugars, and thus very specific antibodies were inhibiting hemagglutination. He found that anti-α-gal antibody was very important and on quantifying the antibody, he found that a very high proportion of antibody, around 1% of circulating IgG was this anti-α-gal antibody (117). It was quite interesting that there was so much antibody of a specific type in humans. Galili subsequently looked at other animal species and found that many did not have this antibody but in fact, these species expressed the corresponding antigen, sometimes in

very high amounts. After extensively studying many animal species, Galili determined that all mammals including new world monkeys (e.g.spider monkey, owl monkey) express the α-gal antigen except for humans, apes (e.g. gorillas, chimpanzees, and orangutans) and old world monkeys (e.g. baboons, rhesus, and cynomologous monkeys) (118-122). Humans, apes and old world monkeys have naturally occurring antibodies against the α-gal antigen (**Figure 1.3**). Though all humans have anti-α-gal antibodies, there is variability in titres and cytotoxicity (123,124). Non-mammals (e.g. birds, fish, reptiles) do not express the α-gal antigen (11).

It was felt that anti-α-gal antibodies developed due to exposure of the host to certain pathogens. Many studies have been done showing that many bacteria (e.g. *Escherichia coli*) as well as parasites express the α-gal antigen (125,126). Galili (119) proposed that there was an evolutionary force (such as infectious agent) which contained the Gal α 1-3 Gal epitope that eventually lead to the inactivation of the α1,3 galactosyltransferase (α1,3 GT) gene in apes, Old World monkeys and humans preventing the expression of αGal on these species' tissues. Indeed, the above animal species do have a α1,3 GT pseudogene that is nonfunctional due to a frameshift mutation (119). This allowed these species to make antibodies to the pathogens to help destroy them and protect the host. Makowka's group (127,128) did a series of experiments which added support to the statement of Galili. They found a monoclonal antibody (of IgM isotype) was able to induce HAR in a hamster to rat model. This antibody was adsorbed against a number of synthetic carbohydrates, some of which were reactive. Further analysis of this monoclonal antibody revealed that it was coded for by a gene in a germline configuration. It is known that similar genes code for

polyreactive natural antibodies directed against infectious organisms. It was thus concluded that anti-porcine Xabs were due to the sensitization of the human host by the carbohydrate structure of the bacterial cell walls and that such Xabs represent a primitive, T-cell independent humoral response to potential pathogens.

When researchers were doing the initial studies of pig-to-human transplantation and pig-to-NHP transplantation, they noticed that the organs were undergoing hyperacute rejection (11,85). It was known that if there was a human ABO blood group mismatch in human-to-human transplantation that a similar process of hyperacute rejection occurred (97). It was also known that this hyperacute rejection was due to the naturally occurring blood group antibodies (anti-A and anti-B) and that these antibodies were glycoproteins and glycolipids. For these reasons, researchers hypothesized that the HAR to porcine organs may be due to antibodies recognizing carbohydrate antigens (129).

A seminal experiment which first identified the anti-α-gal antibody as the important antibody for pig-to-human xenotransplantation was done by Good *et al.* (106). This group perfused 4 pig hearts and 4 pig kidneys with human plasma and subsequently eluted the antibodies from the organs. They tested these antibodies against various carbohydrate antigens of known specificity and they found the strongest reactions to the following antigens: Gal α1,3 gal β 1,4 Glc, Gal α1,3 gal β1,4 GlcNAc and Gal α1,3 gal. An important experiment confirming that α-gal antigen was the important antigen was done by Vaughan *et al.* (130) who demonstrated that transfection of COS cells (a galα1,3gal negative cell line of old world monkey origin) with cDNA for α1,3-galactosyltransferase caused *de novo* expression of α-gal antigen and rendered these cells sensitive to human serum mediated cytotoxicity. In additon, if human serum was

adsorbed against the α-gal expressing COS cells, then this signficantly decreased toxicity of the serum to porcine endothelial cells. A further experiment showing that it was the α-gal antigen that was important was done by Collins *et al.* (108). They found that hearts from new world monkeys (express α-Gal antigen) were hyperacutely rejected by old world monkeys (do not express α-Gal antigen but have anti-α-gal antibody).

Other studies confirming the importance of α-gal antigen include showing prolongation of xenograft survival following depletion of circulating anti-α–gal antibodies (131-135); prolongation of xenograft survival after infusion of α-gal oligosaccharides (136-138) to bind the antibody; and prolongation of xenograft survival after cleavage of α-gal residues from porcine endothelium (139).

Over the past 8-9 years, many studies have been done. The following summarizes some of the important points known about the α-gal antigen and the anti-α-gal antibody:

1. The anti-α-gal antibody recognizes the galα1,3 gal terminal sugar (i.e. gal α1,3gal; gal α1,3gal β1,4Glc-R; gal α1,3gal β1,4 GlcNAc-R (R=glycoprotein or glycolipid)). It is very specific for the α1,3 linkage (98,99,106-111).
2. Anti-α-gal antibody is present in humans, apes, and old world monkeys. Anti-α-Gal antibody is not present in other mammals but instead, these other mammals (eg. pigs) express the α-gal antigen (118-122)
3. The α-gal antigen is quite heavily expressed. It is estimated that there are ~10^6-10^7 α-gal epitopes per endothelial cell (85). Like the A and B blood group antigens on human cells, the α-gal antigen on other mammal's cells is probably involved with functions such as cell signaling, and cell-cell interactions (85).

4. α-gal antigen is also known to be expressed by human tissues in disease states like cancer or autoimmune disease (e.g. Grave's). (140-142) and in hemolytic diseases (112). The α-gal antigen is expressed on senesent red blood cells (116,143,144).
5. Anti-α-Gal antibody is estimated to comprise 4-8% of total IgM (145) and ~1% of total IgG in humans (85). The physiologic purpose of anti-α-gal antibody is believed to be for protection against pathogens such as gut bacteria and parasites (125,126) for protection against cancer (142) and to aid in the identification and removal of sensescent or abnormal RBC (116).
6. The α-gal antigen is variably distributed on pig tissues. McKenzie *et al.* (146) used an α-gal specific biotinylated lectin ($BSIB_4$) and adult pig tissues to determine α-gal antigen expression. Endothelial cells in all small vessels such as capillaries, arterioles and venules stained uniformly and diffusely. Larger vessels like the aorta stained but were less reactive than small vessels. The highest concentrations of staining in organs was in liver and kidney. Liver parenchyma stained uniformly. The brush borders of the proximal convoluted tubules in kidneys stained strongly, the distal convoluted tubles in kidneys stained moderately and there was no staining of collecting ducts or glomeruli (except for the endothelium). Heart muscle was non-reactive although the endothelium on capillaries and other vessels stained strongly. Lung alveoli and bronchioles stained moderately. Other studies (11) have also shown that distribution of α-gal antigen is variable on pig tissues.
7. The α-gal antigen is very similar in structure to the blood group B antigen (85) - see later.

ii) The Complement Cascade

Complement is important in rejection. The complement cascade has two pathways it can follow; the classical pathway and the alternative pathway(147,148). The classical complement pathway involves antibody first binding to antigen on the target cell. The alternative complement pathway is independent of antibody. In this pathway, C3 is directly activated by a foreign antigen and is stabilized by factors B, D, and properdin. C3 then binds with C5 and the rest of the steps are the same as for the classical pathway. Both of these pathways involve the activation of various proteins (usually by the previously activated protein) to ultimately form a membrane attack complex (MAC). This MAC causes pores to form in the cell membrane of the target cell, disrupting its ability to maintain an appropriate intracellular osmotic environment, causing cell lysis. The MAC also mediates other events including secretion of von Willebrand's Factor (149), exposing binding sites for factor Va with a subsequent increase in prothrombinase activity (150); expression of P-selectin which is a platelet and granulocyte adhesion molecule (149) and inducing a procoagulant state (151).

The complement pathway (classical or alternative) activated in xenotransplantation depends on the species combination used (**Table 1.12**). In the porcine to primate combination, it appears to primarily involve the classical complement pathway(152-154)and thus addressing Xabs may be sufficient to address HAR in the pig to human combination. There have been *in vivo* studies showing that complement in the absence of Xab does not cause HAR (154).

iii) Endothelial Cell (EC) Activation

EC cell activation is a very important part of hyperacute xenograft rejection (155). EC normally provide a barrier function and provide an anticoagulant environment. The EC has numerous ways to prevent intravascular thrombosis from occurring. These include inhibiting platelet aggregation by prostacyclin production, nitric oxide generation, ecto-ADPase production (156), thrombomodulin expression and heparin sulfate expression (157). Resting EC do not express VCAM-1 (vascular cell adhesion molecule –1) (158) and E-selectin is also minimally expressed on resting EC (158). EC can be activated in numerous ways. Human Xab alone deliver signals to PEC to induce some, but not all, of the genes associated with full EC activation (159). Adding complement and Xab (by adding human serum) onto PEC will also lead to up-regulation of certain genes for EC activation that are not induced by Xab alone (159). Human natural killer cells (160) and macrophages (*in vitro*, (161))can also activate and lyse PEC (this effect is augmented by adding IgG) (162). EC activation contributes to rejection by causing the loss of barrier and anticoagulant properties (157), and by secreting and expressing various proteins which bring in inflammatory cells. Experiments done labeling EC showed the increased expression of P-selectin, E-selectin (both of which are adhesion molecules for inflammatory cells), and Von Willebrand's factor (which contributes to coagulation) very quickly after graft vascularization. By 48 hours after graft vascularization, there was fibrin deposition, platelet microthrombi (labeled with P-selectin), and the presence of tissue factor and a trace amount of anti-thrombin III, with an absence of thrombomodulin present (155). All of the latter findings suggest a procoagulant environment.

Once the EC has been stimulated, there are a number of events that occur. Nuclear factor κB (NF-κB) is a transcription factor that is essential for the up-regulation of a number of genes associated with EC activation(156). NF-κB is present as a p50-p65 heterodimer in resting cells. It is inactive here because it is complexed with IκB (inhibitor of NF-kB) which keeps the NF-κB heterodimer in the cytoplasm inhibiting it from translocating to the nucleus where it would be involved in up-regulating genes for EC activation (158). When the EC has been stimulated, IκB comes off NF-κB allowing NF-κB to go into the nucleus and increases the transcription of genes encoding adhesion molecules, cytokines (e.g. IL-8) and procoagulant molecules. These changes ultimately result in the EC becoming procoagulant from anticoagulant. Also from the cytokines released and adhesion molecules expressed, there is an influx of inflammatory cells. The occurrence of all these events can be broken down into two phases (i.e. EC activation has two phases). Phase I (or type I activation or "EC stimulation") occurs very early and does not require protein synthesis (156). This phase involves the following: alteration of normal defense mechanisms (such as ADPase, regulators of complement activation (RCA), thrombomodulin, tissue factor pathway inhibitor and heparin sulfate); retraction of the EC; expression of P-selectin/E-selectin; expression of different inflammatory mediators (including C3a, C5a, histamine, PAF thrombin and leukotrienes); leukocyte recruitment; platelet aggregation; and fibrin deposition (100,155,163). Phase II (type II EC activation) occurs later and requires protein synthesis (156). In phase II, there is macrophage and natural killer cell recruitment. There is also involvement of tissue factor, lectins, cytokines (IL-8 and MCP-1) in this

phase (101,163,164). All these events contribute towards the process of delayed xenograft rejection.

B) Preventing Hyperacute Rejection

Knowledge about the mechanisms of HAR and the importance of Xabs, complement, and EC activation have led to many studies investigating methods to overcome HAR. Methods have included: trying to deplete or block the Xabs via various techniques, trying to inhibit complement via various techniques and EC based strategies (trying to modify the α-Gal antigen, as well as modifying complement regulatory proteins on the EC itself).

i) Depletion/Blocking Xenoantibodies

Xab depletion can be accomplished via plasmaphoresis, *ex vivo* perfusion of the recipient's blood through another of the donor's organs or via immunosuppressive drug therapy (152). Xab depletion/blockade is also possible using specific carbohydrate-based immunoadsorptive techniques (165-169). One of these specific therapies is Synsorb 90/Immunosorb B6 (which will be discussed later).

The preliminary experiences gained in removing antibodies prior to transplantation came when investigators tried performing renal allotransplantation across the ABO blood group barrier (170,171). Long term studies adsorbing out antibodies prior to renal transplantation has been successful in preventing HAR in renal allografting over ABO blood group barriers (172). This knowledge of adsorping out antibodies has been applied in xenotransplantation models.

Plasmaphoresis is a method of removing antibodies. Unfortunately, it has the undesirable effect of removing other proteins nonspecifically as well as antibodies. A more specific removal of just the antibodies against the donor organ can be achieved using *ex vivo* donor organ perfusion. Investigators (104) perfused rhesus monkey blood or plasma through a porcine liver and obtained removal of 60-70% of the monkey's Xab against pig antigens. After the perfusion, the complement dependent cytotoxicity was 22 ± 12% (versus 65 ± 6.4% before the pig liver perfusion) and the antibody dependent cell cytotoxicity was 9 ± 6% (versus 33±7% before the pig liver perfusion) demonstrating the effectiveness of donor organ perfusion at removing antibodies of interest. Also, perfusion of a pig kidney with baboon blood demonstrated a decrease in IgM by 68% (173).

Perfusing recipient blood through a different donor organ (e.g. kidney) prior to transplanting another of the donor's organs (e.g. heart) into the recipient does increase graft survival; however the initial perfusion through an organ (e.g. kidney) causes EC activation of the perfused organ as well as other changes. The activated EC release various mediators into the recipient's blood, thus when the second organ (e.g. heart) is transplanted, the blood perfusing it is already "hostile". A solution to this problem is to use immunoadsorbant columns that can pull antibodies out without having the added problems of EC activation. There has been *in vitro* work (174) showing that various immunoadsorbants (such as Synsorb 90/Immunosorb B6) are effective in depleting the α-Gal Xab levels as assayed by α-gal ELISAs. There has also been *in vivo* work in this area. Pascher *et al.*(175) examined the efficacy of highly selective immunoadsorption columns (Therasorb, which are columns consisting of sheep anti-human IgG antibodies

coupled to sepharose CL-4B beads) to deplete xenoreactive human anti-porcine antibodies before *ex vivo* liver perfusion. They found that the Therasorb group had a decrease in IgG of 95% compared to a decrease of 30-39% in all three (IgG, IgM, IgA) in control columns of just the sepharose beads with no sheep anti-human IgG antibodies attached. Leventhal *et al.* (176) studied Xab depletion in porcine-to-baboon xenografts using two different types of columns. The columns they used were the Therasorb columns and an antihuman Ig column (polyclonal antihuman IgM (not chain specific) conjugated to sepharose). The baboons underwent splenectomy 7 days prior to the xenotransplantation. Four days pre-transplant, they had one dose of cyclophosphamide (20 mg/kg IV) and then daily DSPG (4 mg/kg IV). Three days pre-transplant they had horse anti-baboon anti-lymphocyte globulin (20 mg/kg IV), 2 days before transplant they had plasmaphoresis, and 1 day before they had immunoadsorption with the Ig Therasorb column. On the day of the transplant, they had a repeat Ig Therasorb column adsorption immediately before surgery. Post-transplant, they were on a continuous infusion of cyclosporine (3-4 mg/kg/day) and were given 1 dose of 500 mg of methylprednisolone. They found that the Ig Therasorb gave a decrease in IgG of ~97% and a decrease in IgM of ~78-81%. Doing a similar experiment using the IgM columns gave a decrease in IgM of ~83% and a decrease in IgG of only ~13-18%. The xenografts did not undergo HAR and the grafts functioned until the deaths of the animals (from pulmonary infection, hypotension) at 11 and 13 days post-transplant. There was no evidence of rejection on histology. This showed these columns to have efficacy in preventing HAR.

Taniguchi *et al.* (177) used an even more specific immunoadsorption column that actually had the galα1-3gal antigen for *in vivo* immunoadsorption of baboon plasma. They did immunoadsorptions on 3 baboons and looked at pig kidney cell cytotoxicity assays and ELISAs to measure the Xab levels. Two of the baboons had splenectomies done about 1 week in advance of the first immunoadsorption and were put on an immunosuppressive drug regimen from the time of splenectomy which consisted of cyclosporine IV to maintain blood levels >2000 ng/ml, cyclophosphamide IV at 1 mg/kg, and then 0.75 mg/kg twice a day and methylprednisolone IV at 125mg after each immunoadsorption and then at 1 mg/kg IV every 12 hours. Of the 2 baboons that had splenectomies, and immunosuppressive drug treatment, one baboon had immunoadsorption done daily for 4 consecutive days and the other had immunoadsorption done on days 1-3 and days 6, 8, and 13. The third baboon with no splenectomy or no drug treatment, had repeated immunoadsorption treatment. The group found that using this particular immunoadsorption column alone (without splenectomy or immunosuppression), they obtained a significant decrease in Xab levels with a decrease in serum cytotoxicity against pig kidney cells from 100% (pre-immunoadsorption) to 35% (post-immunoadsorption). This baboon had antibody rebound with the antibody levels reaching preadsorption levels. The other two baboons had similar results but antibody rebound was less of a problem.

Our group has done some work with immunoadsorption columns that have the galα1,3galβ1,4Glc antigen bound to insert silica beads. This compound (Synsorb 90/Immunosorb B6 (S90/IB6)) has been found *in vitro* to be effective at depleting the anti-α-Gal antibody in human plasma (174). S90/IB6 at a dose of 0.2g/ml of plasma has

been used in an extracorporeal immunoadsorption (ECI) apparatus to effectively deplete 80% of the anti-α-Gal antibodies from cynomolgus monkeys (178). However, the αGal antibodies rebound to baseline within about 2 days from the ECI and actually overbound to above baseline levels by day 4. Rapamycin at dose of 2.5-4.0 mg/kg given orally once a day is able to prevent the antibodies from overbounding past baseline; however, there is still a significant amount of cytotoxic antibody present that limits xenografting unless other modifications are done.

ii) Complement Inhibition

Another method of avoiding hyperacute rejection is to inhibit complement (179). There are different methods to do this including: giving an agent that inhibits complement, giving an agent that binds complement, and genetically modifying the endothelial cell.

Complement can be inhibited by administering an agent called cobra venom factor (CVF). CVF depletes complement activity through the consumption of C3 (180). In studies in primates (180), one dose of CVF (0.25-0.5 mg/kg) IV or IM caused CH50 (measure of complement activity) to fall to < 5 units and C3 to fall to <8mg/dl and this persisted for about 2-3 days. This fall was associated with a decrease in serum cytotoxicity against pig kidney cells (PK15 cells) from 100% cytotoxicity before CVF treatment, to ~10% cytotoxicity after CVF treatment (180). The study also showed that the administration of CVF alone prevented HAR, but failed to suppress the subsequent vascular rejection that developed over the course of 5-6 days (180). These studies showed that the addition of splenectomy didn't increase survival further, though it did seem to delay the post-transplant rise in anti-α-gal antibody levels (180). Thus, it seems

that CVF is effective in preventing hyperacute rejection. A problem with the use of CVF though, is that CVF has a terminal α-gal residue; thus, repeated doses of CVF treatment evokes a significant synthesis of anti-αgal antibodies (181) which is not desirable.

Another method of inhibiting complement is to treat the recipient with soluble complement receptor type one (sCR1). Prolongation of graft survival has been achieved with this type of treatment (182,183). sCR1 causes the dissociation of the classical and alternative pathway C3 convertases. Studies done treating xenograft recipients with ongoing sCR1 infusions demonstrated prolonged graft survival but rejection still did eventually occur despite the lowered levels of complement (183), and pathology demonstrated extensive deposition of IgM and IgG. If Xabs were depleted by pretreating the recipients with cyclophosphamide, there was a significant prolongation of graft survival (183); however, the animals died or were euthanized (184) secondary to infectious complications. A problem with the use of either CVF or sCR1 is that they interfere with the alternative complement pathway, which is an important defense mechanism against pathogens. Thus, more specific, local targeting would be desirable to avoid the systemic actions of complement inhibition.

The use of genetic engineering helps to give more local effects. Normally, there are natural complement inhibitory proteins on the surface of endothelial cells which protect the cell from lysis. These regulators of complement activation (RCA) include the proteins: decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and homologous restriction factor (HRF, CD59) (185). These regulators of complement activation function in a species-specific manner and are believed to be

important in xenotransplantation (186). The reason they are important in xenotransplantation is that the pig regulators of complement activation on pig endothelial cells will likely be less able to protect the pig endothelial cells from human complement (186) since the regulators are species-specific. With this knowledge, investigators have employed genetic engineering techniques to express human RCA on porcine endothelial cells (PEC) (185,186). PEC expressing human DAF have been created and the DAF causes the splitting of C3 convertase which has been formed as a part of the complement cascade initiated by human anti-αgal IgM binding the EC. PEC expressing MCP have also been made and the MCP inactivates C5 convertase. Lastly, PEC expressing human CD59 have been made and the CD59 blocks the formation of the membrane attack complex (185). Transgenic pigs have subsequently been transplanted into nonhuman primates with interesting results. Transgenic pig (expressing human CD59) hearts have not had a significantly prolonged survival. When compared histologically, to normal porcine hearts, transgenic cardiac endothelium has reduced deposition of C5b and MAC, but does not have decreased binding of C3. Other groups have also used pigs transgenic for human DAF (187-192) and found that these hearts transplanted into cynomolgus monkeys can survive greater than 21 days if heavily immunosuppressed. Some of these monkeys have needed to be sacrificed because of complications of the immunosuppression, but at the time of sacrifice the xenografts were still functional and histology of the heart revealed no significant immunoglobulin or complement deposition (192). Criticisms of these experiments (using transgenic pigs) are that the hearts used actually expressed only low levels of αgal on their endothelium, that one still needed heavy immunosuppression which lead

to side effects, and that if no immunosuppression was used, the transgenic hearts survived a median of about 5.1 days (188).

Another group (193) also found that heavy immunosuppression was needed with transgenic pigs. They found that hearts from pigs transgenic for human DAF were not hyperacutely rejected when heterotopically transplanted into cynomolgus monkeys, however prolonged survival needed immunosuppression with cyclosporine A, cyclophosphamide, and methyl prednisolone. The above studies are important because they demonstrate that transgenics do prevent hyperacute rejection; however, grafts still reject unless heavy immunosuppression is used.

Thus, complement inhibition can prevent HAR but one still later runs into further antibody mediated rejection.

iii) Endothelial Cell Modification

The next way of preventing HAR includes EC based strategies. There are numerous manipulations that have been done in this area, but we will concentrate mostly on some work that has been done in regards to modifying the α-gal antigen on the EC, and modifying EC activation. (Another EC based strategy-expression of human complement regulatory proteins on PEC has already been discussed in the previous section).

As has already been mentioned, the α-gal epitope is expressed on the vascular endothelium of many species (including pig) because of the presence of a functional α1,3 galactosyl transferase enzyme. It has also been mentioned that the primary epitope of importance known at present in HAR in the pig-to-human model is the α-gal epitope. Thus, investigators rationalized that if this epitope could be genetically "knocked out"

or modified on pig endothelium, then this may prevent HAR. α-gal expression can be [redacted]minated in "knockout" mice and HAR can be prevented in this model (11). Unfortunately, in a larger animal model (like the pig), the technology to knock out the gene for the α1,3 galactosyl transferase enzyme is not readily available at present. Thus, investigators (11) have created transgenic pigs which have increased expression of the fucosyl transferase enzyme and therefore, there is more H antigen (blood group O antigens) formed than α-gal antigens formed. Use of these transgenic pigs has shown some benefit but no significantly prolonged xenograft survival.

Since EC activation and death plays a crucial role in xenograft rejection, ways to modify this has been attempted. As described earlier, the ubiquitous transcription factor NF-κB is felt to be central in activating the genes involved in EC activation (194). It has been found that the agent pyrrolidine dithiocarbamate (PDTC) inhibits NF-κB activation and inhibits E-selectin, IL-8 and tissue factor expression (156,195). Another method of inhibiting NFκB is to over-express IκBα in the cytoplasm (196). IκBα is a natural inhibitor of NF-κB and when it is bound to NF-κB, it keeps NF-κB from migrating to the nucleus where NF-κB would lead to the transcription of EC activation genes. The use of a recombinant IκBα adenovial vector to transduce PEC suppressed the transcription of various EC activated genes (195). The above methods again have been tried in xenotransplantation with limited success.

Another method of addressing the EC in xenograft rejection is to focus more peripherally on the adhesion molecules. The use of NPC 15669 – a drug which inhibits neutrophil adhesion to the EC of the donor has been used combined with complement inhibition in small animal models of discordant heart transplantation with prolonged

xenograft survival (197). P-selectin is an important adhesion molecule for platelets and neutrophils, and experiments using antibodies against P-selectin as well as a PAF antagonist, led to prolonged xenograft survival in a rat model (198). Investigators have also used monoclonal antibodies against porcine VCAM to address the EC issue in xenograft rejection.

Another method of dealing with the consequences of EC activation (intravascular thrombosis) is to address the issues of thrombosis directly. The use of GPI 562 (a gpIIbIIIa antagonist) has been used to inhibit platelet aggregation with some results (199); and 5D2 MTH 958 (a thrombin inhibitor) has been used in an isolated perfused heart model (200). All of the above methods have shown that their potential applicability to clinical transplantation exists but that further research is needed.

The next method of dealing with EC death (apoptosis) is in regards to the alteration of expression of various genes for endothelial cell survival. There are genes which if expressed by the EC are "apoptotic" and these include Bad, Bax, and BcL-XS (201). There are also genes, which if expressed are "protective" or "anti-apoptotic" and these include A20, Bcl, Bcl-XL and hemoxygnase (201). A20 is a novel zinc finger protein and is a tumor necrosis factor (TNF) inducible gene product in human umbilical vein cells (202). One of the main functions described for A20 is its ability to protect cells from TNF-induced apoptosis, though the mechanism of how this occurs is still unknown (202). It is felt that the inhibitory effect of A20 is related, at least in part, to the inhibition of NF-κB at a level upstream of IκBα (202). Experiments done cotransfecting bovine aortic EC with an A20 expression vector together with different reporter constructs such as E-selectin, IL-8, IκBα and tissue factor reporters, totally inhibited their induction on

stimulation with TNF or LPS (202). Specifically, Ferran et al. (202) transduced PAEC using an adenovirus vector with A20 and found that these PAEC, when stimulated with TNF (100U/ml), LPS (100ng/ml) human thrombin (10U/ml) or 10^{-8} mol/L phorbol ester, had a 60-90% decrease in E-selectin, IL-8 and IκBα mRNA (via Northern blot analysis) (202). Thus, there are potential ways to alter EC to help with xenotransplantation.

All of the above endothelial cell strategies have potential but more research is needed.

C) Acute Vascular Rejection/ Delayed Xenograft Rejection:

Once HAR (rejection within minutes to hours) has been prevented, discordant xenografts undergo the next phase of the rejection process termed acute vascular rejection by Platt (203) or delayed xenograft rejection by Bach (204). This phase of the rejection process has some important characteristics which include: antibody production and deposition causing damage; further activation of the EC which leads to thrombosis, and the infiltration of various immune cells (particularly natural killer NK cells and macrophages) (203,205-207). Accelerated rejection occurs days after the transplant (208) and it occurs in discordant combinations such as guinea pig to rat and pig to nonhuman primate (once HAR has been avoided) as well as occurring in concordant combinations. Methods to address "accelerated rejection" include immune suppression (by using total lymphoid irradiation, splenectomy and/ or immunosuppressive drug therapy); and tolerance induction (207,209-211). For immunosuppression methods, immunosuppressive drugs are likely the best method to use as they can more specifically target immune cells. Important points include that both T cells and B cell immunosuppressive drugs should be used in combination (11,207). Tolerance induction

involves teaching the recipient's immune system that the donor's antigens are self antigens (148). If this can be done, the organ can be transplanted without the need for any immunosuppression (212,213). Work in tolerance induction in baboons is being conducted in Boston (209). Initial results are encouraging.

Overall, many studies have been done in rejction (espeically HAR). However, no one has looked at the effect of specifically depleting the anti-α-gal antibody on survival of pig working hearts. Hearts are different from kidneys physiologically and thus they need to be evaluated seperately (see discussion in chapter 5 for more details).

V. Human ABO Blood Groups

There is similarity between the α-gal antigen and the A and B antigens (see **Figure 4.1(a)**. In fact, it is felt that the isohemagglutinins (anti-A and anti-B antibodies) and xenoantibodies are members of a distinct family of natural antibodies (214). In allotransplantation, if one has ABO incompatibility between donor and recipient, then hyperacute rejection (similar to that of pig-to-human xenotransplantation) can occur and it is usually caused by anti-A and anti-B antibodies (11). For all of these reasons, one may suspect that there may be a human ABO blood group difference in porcine-to-human xenotransplantation (though many in the xenotransplant community feel that human ABO blood groups do not matter in porcine xenotransplantation) (11,13,215).

Before progressing, some background information about human blood groups is warranted. Differences in blood groups between species was the first to be discovered before blood groups within a species. Landois (216) in 1875 discovered that mixing the red cells of one animal with the serum of another and incubating the mixture at 37°C lead to red cell lysis. This was the first demonstration of blood group incombatibility. Landsteiner (217), in 1900 used this same method to find differences within humans – the ABO blood group system – and he received the Nobel Prize in 1930 for this work (148). Over the next many years, there were more blood groups identified, and to date, there are over 15 different blood group systems known to exist in man: ABO, Rh, Lewis, MN, P, Kell, Duffy, Kidd and others (216,218). Some of these blood group systems have naturally occurring antibodies in them and others do not; however, only the ABO system is considered clinically important in terms of naturally occurring antibodies as this is the only system where the anti-A and anti-B antibodies are active at warmer temperatures

(216). The other systems' naturally occurring antibodies generally are active at cooler (4°C) temperatures.

There are some hypotheses on the reason for the emergence of the different ABO blood types. Anthropologists and other researchers believe that the first blood type to exist in Neanderthals in Africa was blood type O dating back ~50,000 B.C. (219). This blood type suited the diet and environmental conditions of these hunter-gatherers (primarily meat eaters) allowing them to survive and thrive. As "big game" food became scarce, people moved to other parts of the world. With new living conditions, people's diets and environmental conditions changed. As an adaptation to the new diet (which was more agricultural in nature); and the new environmental agents, blood group antigens changed and so did blood group antibodies (220). This process of adaptation to new environments and diets is believed to have resulted in the emergence of blood groups A and B from O about 15000 to 25000 years ago. As intermingling between different people occurred, blood groups AB formed about 2000 to 3000 years ago (221,222).

Because pressures related to geographic location led to changes in blood type, many ethnicities have different blood group distributions (216). Currently it is felt that there are a number of types of people based on blood group. The most common blood type is blood group O. Certain populations (such as Arabs in Iraq) have very high numbers of O individuals. Blood group A individuals tend to be present in Western Europe and their frequencies decrease the more east one goes. Areas around the Mediterranean, Adriatic and Aegean seas, particularly in Corsica, Sardinia, Spain, Turkey and the Balkans have a high denisity of A people. Blood group B stretches as a belt across the Eurasian plains down to the Indian subcontinent. Japanese, Mongolian,

Chinese, Koreans, and Indians have a higher percentage of B individuals in their populations than other groups. Blood group AB is a mixture of different peoples. (216,221).

Some people believe that certain blood groups are more prone to certain diseases partly because of their ancestry (223). As environmental conditions caused the emergence of the different blood groups, some feel that if people do not live their lives or eat the type of foods that their ancestors did (i.e. the right kind for their blood type), then they will become ill (223). Some diseases that tend to be prevalent in the different blood groups include: blood group A and stomach cancer (224) and higher levels of clotting factor VIII in blood group A versus O (225).

Returning to the ABO system, the A, B, and H antigens are glycoproteins/glycolipids, like α-gal (85). Similar to α-gal which is the enzymatic product of the α-1,3 galactosyl transferase (α-1,3 GT) enzyme, the A antigen is the enzyme product of the α-3-N-acetyl-D-galactosaminyl transferase (A transferase) enzyme and the B antigen is the enzymatic product of the α-1,3 galactosyl transferase (B transferase) enzyme (85,216). Though both α-gal and B seem to have same enzyme, they are distinct, as B transferase needs fucose in order for it to work (85). All individuals carry the H transferase which gives the expression of the H antigen on tissues (216). If one has the gene for the A transferase, then one will add GalNAc to H antigen to make the A-antigen and will be blood group A. If one has gene for the B transferase, then one will add Gal to H antigen to make B antigen; and will be blood group B; if one has both genes then one will express both A and B antigens and be blood group AB; and lastly, if one has neither gene, one will just express the H antigen and be blood group O (85). As

mentioned earlier, the mammals that don't express the α-gal antigen correspondingly express the anti-α-Gal antibody (11,13,85). In a similar fashion, individuals who are blood group A will have naturally occurring anti-B antibody; those who are blood group B will have naturally occurring anti-A antibody; those that are AB will have neither anti-A nor anti-B antibody and those that are O will have both antibodies. The A, B and H antigens (like the α-gal antigen) are believed to be involved with cell signalling and cell-cell interaction, since almost all of the A, B, H antigen tends to be bound to band 3 in red blood cells that acts as a major ion channel (216). Lastly, similar to the anti-α-gal antibody, the anti-A and anti-B antibodies are felt to have arisen to protect one against pathogens (particularly those colonizing the bowel) (85).

There is a lot of similarity in antigen structure between the α-gal antigen and the B antigen (**Figure 4.1a**). The main difference between the two is the presence of a fucose residue on the second galactose in the B antigen. It is known that even though an individual B cell clone makes a monoclonal antibody to a specific antigenic determinant, there are many different B cells that recognize a particular antigen and make antibodies to different antigenic determinants. In addition an antibody can cross react with a similar antigen (148). Galili (85) found that anti-B antibody would cross react with the α-gal antigen. Galili has also found that individuals who are blood group A and O have anti-α-Gal antibodies that will cross react with the B antigen; however anti-α-Gal antibodies from individuals who are blood group A and AB do not cross react with the B antigen. This precise specificity of the anti-α-Gal antibody in blood group B and AB people is important to avoid autoimmune disease (85). Also, since we know that there is still some (though less than anti-B) similarity between α-gal antigen and A antigen (A antigen has 2

sugars different from α-gal antigen), there may be some cross reaction between the anti-A antibody and the α-gal antigen (though the cross reactivity is likely to be less than that of anti-B antibody). Due to the different degrees of similarity between the α-gal antigen and the B and A antigen; one may hypothesize that the anti-B antibody may augment rejection whereas the anti-A antibody may act as a competitive inhibitor since it has more structural dissimilarities to the α-gal antigen (**Figure 4.1b**). Based on this reasoning, one would say that there should be a blood group difference in survival of pig-to-human xenotransplantation. There have been some groups that have looked at blood group issues in porcine to human xenotransplantation (215,226-229). All of these groups have looked at *in vitro* assays (binding assays, ELISAs, cytotoxicity assays) and some have found blood group differences whereas others have not. Many were finding trends but not statistically significant results due to lots of variability in their data. Eventhough there is evidence (11,85,227,228) in the literature that anti-B antibody can bind to α-gal antigen and to pig tissue, the xenotransplantation literature states that at present, it does not appear that human ABO blood group has a role in porcine-human xenotransplantation (11,13). The reason for this statement may be because it has been difficult to consistently show significant cytotoxicity of the different blood group to porcine cells. The reason for this difficulty may be due to the assay system used and due to sample sizes (see further discussion in chapter 4). If one was to use larger sample sizes and a sensitive organ function system, then subtle, yet important differences may be discernable. A porcine working heart model may do this.

VI. Models to Study Hyperacute Rejection

Models to study hyperacute rejection (HAR) include *in vitro* models, *in vivo* models, and *ex-vivo* models. Some of the *in vitro* models consist of hemagglutination/hemolysis assays, cytotoxicity assays and assays to measure changes of cells in culture (eg. expression of proteins, permeability changes, etc.). Specific examples of *in vitro* models would include looking at the hemagglutination/hemolysis of porcine red blood cells (RBC) caused by human serum. The effect of human serum on the survival of pig endothelial cells (EC), such as in a MTT cytotoxicity assay, would be another example of an *in vitro* cytotoxicity model of HAR. Measuring the amount of P-selectin expression on pig EC caused by human serum may be an example of an *in vitro* system designed to measure changes in cells. Some of the advantages of *in vitro* systems include the fact that they are relatively inexpensive; one can do many replicates relatively easily; one can test many different variables in isolation and in various combinations to see the effects on the cells to help determine mechanisms; one can study cellular interactions, and one can do molecular biology investigations on the cells. Some of the major disadvantages of *in vitro* systems include that cells in culture (e.g. petri dish in incubator) may not look or function like a normal cell in its natural environment and thus the knowledge gained from *in vitro* data may not be directly applicable to *in vivo* situations. Humans are organ systems in which there is an enormous amount of interaction between cells of the different systems. Isolated cells *in vitro* are not exposed to all these other influences; thus, there may not be an accurate picture of *in vivo* activities. *In vitro* systems may not give an accurate picture of entire organ function.

Also, cells in culture may be a lot stronger and able to withstand more deficiencies compared to an organ. *In vitro* systems may be a less sensitive system for finding subtle differences between various groups. For example, a heart needs regular oxygenated perfusate and precise conditions to continue to contract and do mechanical work to pump blood. Any prolonged ischemia may detrimentally effect the heart; however, myocytes or endothelial cells may be able to stand more injury (temperature, acidosis, ischemia), making it hard to find subtle differences between various test groups.

In vivo models of HAR include transplanting organs from one species to another. There are many small animal models that have been used for xenotransplantation (230,231). Advantages of these models include their relative inexpensiveness compared to large animal models. However, some small animal models such as pig-to-rabbit or mouse-to-rat are not directly relevant to humans, as neither has anti-α-Gal antibodies which are of critical importance in the pig-to-human combination (11,85). Gal knockout mice are useful to gain some insight as they make anti-α-gal antibodies (232,233); however, the focus in human xenotransplantation is on organs from larger animals such as the pig. Pig organs are antigenetically different from mouse organs with mouse organs having more non-α-gal antigens that humans can have antibodies to (232). Direct information about the pig-to-human combination is difficult to get from knockout mice. HuSCID-beige mice basically have a human immune system (148) but are again not as directly useful for the pig-to-human combination as one cannot transplant a pig heart into one of these mice. In addition, the complement proteins that these mice would make would be mouse complement from mouse liver and not human complement.

A larger *in vivo* model such as pig-to-non-human primate (NHP) has many advantages. Some NHP are fairly close to humans and thus should, for many things, be a good predictor for what should happen in a human (11). Also, like in small animal models, being *in vivo* allows all the interactions that are going to occur between cells and organs to occur. However, there are significant disadvantages to using a large animal model. Many of these relate to cost issues (11). Working with primates requires considerable resources such as biocontainment housing, operating rooms, specifically trained personnel, an ICU/recovery room, and monkey blood products, just to mention a few. In addition, by using NHP one cannot often have a lot of replicates and cannot test a lot of variables as this would generally require more NHP to be used. There may be individual differences in the NHP so that the results from one animal may not be representative of other animals. Another very important disadvantage of using NHP for xenotransplantation studies is that some NHP do have significant differences compared to humans (234,235). Baboons are commonly used in xenotransplantation studies (11); however, the dissimilarity index **(table 1.9)** shows that old world monkeys (like baboons) are quite dissimilar to humans. Very importantly (for this project), some NHP (like baboons) do not have the exact same blood types and blood type distribution as do humans. Most humans are blood group O; however <2% of baboons are blood group O (234). The blood group distribution in baboons is: 1/6 - 1/3 are blood group A, 1/3 - 1/2 are B and 1/3 are AB (234). Thus, large animal *in vivo* models do have significant disadvantages.

The main model chosen for this research project is an *ex-vivo* porcine working heart model. The advantages of an *ex-vivo* working heart model include the fact that it

has some physiological similarites (preload, afterload) to what normally occurs *in vivo*. One is able to measure cardiac outputs, blood pressure and heart rate, and then subsequently calculate various indices of cardiac function. As the model is *ex-vivo*, one can easily obtain samples from different parts of the heart (eg. aorta, coronaries, pulmonary artery) to look for differences in various factors in different parts of the heart - this would be harder in an *in vivo* system. As there are no primates being transplanted, the procedure is easier to do technically and practically, requiring less resources, less personnel and thus, less cost. One can do many replicates using this model increasing confidence in the findings and many different variables can be tested. Factors can be studied in isolation (such as the effect of removing antibody) or interactions of various factors can be studied. More precise manipulation of things such as heart rate (by pacing), afterload, and preload pressures is possible as is the ability to use various perfusates such as blood, plasma, or buffer.

A very important advantage of this system is the ability to test human blood in it. As mentioned earlier, there are important differences between some NHP and humans such that data gathered from NHP experiments may not be directly extrapolatable to humans. In the *ex-vivo* system, one can directly test the effects of the various components of human blood on the hearts. Additionally, if one is interested in a specific question (e.g. blood groups), using an *ex-vivo* model allows one to test pooled human plasma (where individual human differences hopefully become negated and the factor in question predominates).

The disadvantages of an *ex-vivo* system are that the hearts cannot be maintained for more than "the hours range". The probable reasons that these hearts do eventually fail

is that waste products cannot be removed because of the lack of a liver or kidney in the circuit. The potassium level increases while the pH decreases over time. Because of this relatively short survival time, one cannot study factors that would normally take place after this time period, such as factors related to acute vascular rejection. Another disadvantage of an *ex-vivo* model is that the various interactions that would occur between different systems in an *in vivo* model cannot be examined properly. For the questions we want to answer, an *ex-vivo* porcine working heart model is ideal.

There are two types of isolated heart perfusion systems. There is a non-working mode (i.e. retrograde or Langendorff) perfusion system and a working mode system. The Langendorff system was initially developed by Oscar Langendorff in 1812 when he used cat hearts to determine that the heart gets its blood supply from the coronary arteries (236). In a non-working heart preparation, perfusate enters the coronary circulation via gravity and nourishes/oxygenates the myocardium causing the heart to beat. Thus the heart will beat but there is no blood in the left ventricle which the heart has to eject against a resistance. One can thus not measure cardiac output in a non-working heart system, though one can measure heart rate and ventricular pressures. Not being able to measure cardiac outputs is a very significant drawback of the non-working system.

In a working system, perfusate enters the left atrium and then goes into the left ventricle where it is ejected against an afterload resistance to be recirculated throughout the system. The metabolic demand of a working heart is very high and in this system, one can measure blood pressure, heart rate, and cardiac output. Coronary perfusion is now dependent upon good left ventricular function (and not gravity) as left ventricular ejection at the aorta will determine the amount of coronary perfusion. The working mode

is thus more sensitive to injury, is a more physiologic system, and enables measurements of important variables like cardiac output when compared to a non-working heart model preparation. For the above reasons, a working heart model was used instead of a non-working heart model.

There have been some studies by a limited number of groups looking at hyperacute rejection of pig hearts with human blood using a porcine working heart model (200,237-246). Fodor *et al.* (240) looked at hDAF transgenic pig hearts compared to normal pig hearts perfused with human blood. They did not find a survival difference between their groups but found that the transgenics had better function at certain time points compared to their controls and that certain markers of EC activation (eg. $PGF_{1\alpha}$) were lower in the transgenic hearts compared to controls. This group also did not have a negative control (autologous pig blood perfusing its own heart) group. Brenner *et al.* (237) have used Therasorb-Ig immunoadsorbent columns to remove a large percentage of total antibody (IgM and IgG) and complement from human blood and then have used this depleted blood to perfuse working hearts. This group found improved survival of their working hearts compared to controls; however, in clinical practice, one will not want nonspecific significant depletion of total antibody and complement as this leaves the recipient more vulnerable than necessary to infection and cancer (11). Others have looked at complement inhibition (238,239,243,246); while others have investigated coagulation inhibition (200) in their pig working hearts. Many of these manipulations did show some benefit; however, many of these models either did not have a valid model (as could be determined by the model presented in the paper); either did not have a well functioning model (re. cardiac function over time); either had a circuit volume that was

very large (making it difficult to get enough blood to fill the system) or had other concerns. Thus, there were concerns with some of the models in the literature. In addition, no one had specifically examined the role of human ABO blood group and specific anti-α-gal antibody depletion in pig working hearts perfused with human blood. We felt this was a gap in the literature that needed to be filled.

VII. Rationale

Reviewing the literature, the following questions had not been adequately addressed. Given the similarity of the α-gal antigen to the B antigen, is there a human ABO blood group difference in porcine–to-human cardiac xenotransplantation? Secondly, does the Synsorb 90/ Immunosorb B6 (S90/IB6) immunoadsorbent specifically deplete just the anti-α-gal antibody or does it deplete other factors as well? Does specific depletion of the anti-α-gal antibody prolong survival of porcine working hearts perfused with human blood? These are important questions to answer due to their clinical implications. Firstly, if there is a blood group difference, then this may suggest that modifications in treatment (e.g. more or less immunosuppression) may be justified for certain individuals. Secondly, the immunoadsorbent S90/IB6 may not specifically deplete anti-α-gal antibody making it the same as a product already in existance – Therasorb-Ig. Thirdly, S90/IB6 may not be effective when using human blood and a pig working heart. If this is true, then it suggests that different organs need different immunoadsorption protocols.

We hypothesized that:

1) Blood groups with the anti-B antibody (ie. blood group A and O) would have more *in vitro* and quicker *ex-vivo* porcine xenograft rejection compared to the blood groups not having the anti-B antibody (ie: blood group B and AB).

2) S90/IB6 would specifically deplete anti-α-gal antibody from human blood and this blood perfusing pig working heart would have longer survival compared to blank adsorbed human blood perfusing pig working hearts.

The specific objectives of this study were:

1) To develop and optimize an *ex vivo* porcine working heart model.

2) To develop and validate this *ex vivo* model as a porcine to human hyperacute rejection xenotransplantation model.

3) To determine if there is a human ABO blood group difference in regards to porcine xenograft rejection (*in vitro* and *ex vivo*).

4) To determine the specificity of adosrption with S90/IB6 immunoadsorbent and to determine if S90/IB6 immunoadsorbed human blood can prolong xenograft survival.

VIII. Figures and Tables

Table 1.1 Data on organ transplantation activities in 1999 (7)

Transplant Organization	Countries	Population x 10^6	Cadaveric Donors	Number per 10^6 Population
ET	Germany, Belgium, Austria, Luxembourg	114.5	5593	48.8
UKTSSA	United Kingdom, Republic of Ireland	62.88	2624	41.7
UNOS	USA	268.0	17644	65.8
CORR	Canada	30.5	1363	44.7

ET=Eurotransplant, UKTSSA=United Kingdom Transplant Support Service Authority, UNOS=United Network for Organ Sharing, CORR=Canadian Organ Replacement Register

Table 1.2 Transplant activity in the United States (12)

Organ	Number Waiting (as of Nov. 4, 2001)	Transplants Performed (in 1999)	% Transplanted
kidney	45978	12483	27
liver	16468	4698	29
heart	4120	2185	53
lung	3636	885	24
heart-lung	217	49	23
Total	**71419**	**20300**	28

Table 1.3 Cadaveric organ donor information for the year 1999 by method of consent.(16)

Method of consent	Countries	Total population	Total cadaveric organ donors	Number of cadaveric organ donors per 10^6 population
Presumed	Finland. Portugal, Austria, Sweden, Czech Rep., Slovak Rep., Hungary, Poland	96.5×10^6	3370	34.9
Theoretically presumed	Spain, Italy, Greece, Belgium, France	177.7×10^6	9771	55.0
Informed	USA, Canada, UK, Ireland, Denmark, Netherlands, Germany	462.7×10^6	25925	56.0

Presumed consent does not increase the number of cadaveric donors

Table 1.4 Reported clinical attempts at renal xenotransplantation in humans (84)

Date	Surgeon	Donor	(n)	Graft Survival
1905	Jaboulay	Pig/Goat	1	3 days
1910	Urger	Goat	1	3 days
		Monkey	1	<2 days
1923	Neuhof	Sheep	1	9 days
1963-64	Reemstma	Chimpanzee	12	<9 months
		Monkey	1	10 days
1964	Hitchcock	Baboon	1	5 days
1964	Starzl/Millard	Baboon	6	<2 months
1964	Hume	Chimpanzee	1	1 day
1964	Traeger	Chimpanzee	3	2 months
1965	Goldsmith	Chimpanzee	2	4 months
1966	Cortesini	Chimpanzee	1	1 month

Table 1.5 World experience in clinical heart xenotransplantation (61)

Year	N	Surgeon	Institution	Donor	Type of transplant	Outcome
1964	1	Hardy	University of Mississippi, Jackson, Mississippi, USA	Chimpanzee	OHT	Functioned 2 hours (heart too small to support circulation)
1968	1	Ross	National Heart Hospital, London, UK	Pig	HHT	Cessation of function within 4 minutes (? vascular function)
1968	1	Ross	National Heart Hospital, London, UK	Pig	Perfused with human blood but not transplanted	Immediate cessation of function (? vascular function)
1968	1	Cooley	Texas Heart Institute, Houston, Texas, USA	Sheep	OHT	Immediate cessation of function (? vascular rejection)
1969	1	Marion	Lyon, France	Chimpanzee	? OHT	Rapid failure (? raised pulmonary vascular resistance)
1977	1	Barnard	University of Cape Town, Cape Town, South Africa	Baboon	HHT	Functioned 5 hours (heart too small to support circulation)
1977	1	Barnard	University of Cape Town, Cape Town, South Africa	Chimpanzee	HHT	Functioned 4 days (failed from probable vascular rejection)
1984	1	Bailey	Loma Linda University, Loma Linda, California, USA	Baboon	OHT	Functioned 20 days (failed from vascular rejection)
1991	1	Religa	Silesian Academy of Medicine, Sosnowiec, Poland	Pig	OHT	Functioned <24 hours
1996	1	Baruah	India	Pig	OHT	Functioned <24 hours

Table 1.6. Reported clinical attempts at hepatic xenotransplantation in humans(11)

Date	Surgeon	Donor	(n)	Graft Survival
1966	Starzl	Chimpanzee	1	<1 day
1969	Starzl	Chimpanzee	2	<9days <2 days
1969	Bertoye	Baboon	1	<1 day
1970	Leger	Baboon	1	3 days
1970	Marion	Baboon	1	<1 day
1971	Poyet	Baboon	1	<1 day
1992-93	Starzl	Baboon	2	<70 days
1993	Makowka	Pig	1	1 day

Table 1.7 Clinical attempts at extracorporeal xenograft perfusions(61)

Organ	Year	Investigator	N	Location	Patient population (indication)	Outcome	Comments
Bioartificial liver dialysis machine (encapsulated porcine hepatocytes)	1990s	Circe Biomedical	52	USA	Hepatic Failure	-some improvement in hepatic encephalopathy and some biochemical parameters -no signs of synthtic activity reported	-Phase I and Phase II trial results
as above		as above	~60	Multicenter (10 USA, 9 European)	as above	no results at present	-Phase III trial (placebo controlled) - curently ongoing
Similar device to above	Began in Nov. '98	Excorp Medical	6	University of Pittsburgh, USA	as above	"no unanticipated adverse effects observed"	-Phase I trial -plan to have total of 15 in study and 30 reference patients.
Extracorporeal nontransgenic pig liver perfusion	late 1990's	Researchers at Duke University	7	Multicenter (5 Duke University, 2 Medical University of South Carolina, USA)	as above	"transient improvements in several of the patients"	
Extracoporeal transgenic (hCD55 and hCD59) pig liver perfusion	1997-1998	Goran Klinmalm (pigs from Nextran)	2+	Dallas, USA	as above	able to bridge to allotransplantation	
Extracorporeal pig/kidney perfusion	1994	Researchers in Sweden	2	Sweden	Renal failure patients already on dialysis	1 pt→ kidney rejected after 1 hour 1pt→ anaphylactic reaction and experiment stopped	plasmaphoresis prior to kidney perfusion

Table 1.8 Clinical attempts at cellular xenotransplantation (61)

Transplant Tissue	Year	Investigator	N	Location	Patient Population (Indication)	Outcome	Comments
Encapsulated neonatal bovine chromaffine cells into spinal canal	Phase I → 1990's	Cytotherapeutics Inc. (American Biotech Co.)	?	Europe and USA	Severe pain in patients with advanced cancer and neurologic pain	- some benefit at implantation site (lumbar and sacral region) - safe	- no immuno-suppression - some technical problems
Same as above	Phase II → 1998-1999 (blinded placebo controlled study)	AstraZeneca	~85	Poland, Czech Republic, and Switzerland	Similar to above	- insufficient efficacy of treatment but no serious adverse events	- no immuno-suppression - cells survived well and in many cases improved - no further studies planned at present
Encapsulated transgenic (producing a neurotrophic growth factor) fetal hamster cells into spinal canal	1990's	Dr. Patrick Aebischer	6+	Switzerland	Amyotrophic lateral sclerosis	- Significant levels of growth factor detected in spinal fluid but no reports of clinical amelioration of symptoms so far	
Fetal porcine dopaminergic neurons ($12x10^6$ cells injected unilaterally into putamen & caudate)	Phase I→ started 1995	Diacrine/ Genzyme USA	12	USA	Parkinson's disease (?resistant to medications)	- some benefit? - no safety problems	- 6 got cyclosporine - 6 got porcine cells transplanted with antibodies to MHC Class I
Fetal porcine dopa neurons (⅓ of patients got bilateral implants of $25x10^6$ cells and ⅓ got sham)	Phase II → 1999 (placebo controlled double blind)	Same as above	18	Boston, New England, Atlanta, Georgia, Tampa (USA)	Same as above	- Mar.16/2000→ announcement that 18 mo. after procedure, both treated & controlled group showed improvement – strong placebo effect	- Phase III trials postponed until further data analysis done on Phase II data - Cyclosporine used in all patients
Fetal Porcine GABA neurons	Phase I→ late 1990's early 2000	Same as above	12	USA	Huntington's chorea	- no clinical improvement but 3-4 of 12 transplanted early in disease have stabilized	- Phase II trial being discussed

(Continued next page)

Table 1.8 (cont'd) Clinical attempts at cellular xenotransplantation

Transplant Tissue	Year	Investigator	N	Location	Patient Population (Indication)	Outcome	Comments
Porcine GABA neurons	late 1998		3	USA	Focal epilepsy requiring surgical therapy	- good effect in one patient such that surgery not needed - other 2 patients did not benefit and needed surgery	- another 3 patients planned in trial & FDA has approved a trial in multifocal epilepsy
Fetal porcine neurons	Started Sept. 1999	Diacrine	5	USA	6 months to 10 years after stroke with stable deficits	- 4 improved (especially with respect to facial palsy)	- 2 had seizures thus trial halted until more info on cause of seizure is determined
Nonencapsulated fetal porcine islets	1990s	Carl Groth & Claes Hellerstrom	10	Sweden	Diabetes mellitus (patients also had renal defects)	- graft survival in 5 patients - no patient could stop exogenous insulin injections	
Encapsulated porcine islets	1990s	Dr. Elliot in collaboration with Vivorex (American Biotech Co.)	6	New Zealand	Diabetes mellitus	- results not reported so far	
Fetal rabbit islets	1990s	Professor Shumakov	several hundred	Russia	Diabetes mellitus	- results not reported so far	
Fetal pancreatic tissue from pigs & calves	1990s	?	?	China		- results not reported so far	
Baboon bone marrow	1996	Suzanne Ilstad	1	Pittsburgh, USA	Advanced HIV	- microchimerism for first 13d after transplant then rejected?	

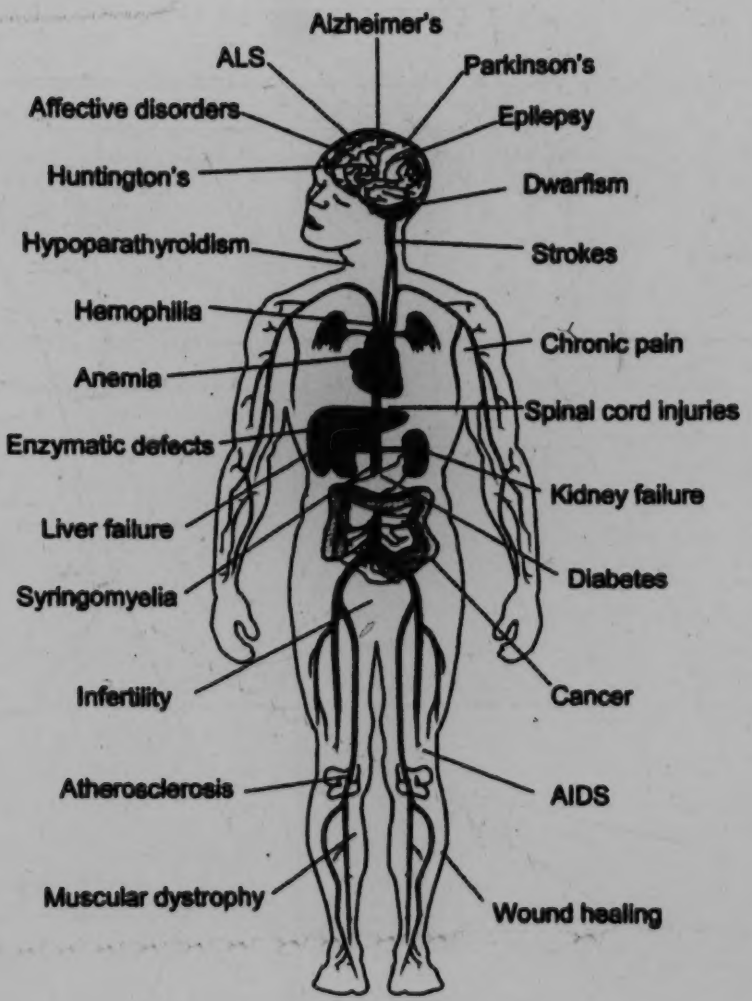


Figure 1.1 Medical disorders potentially treatable by the transplantation of animal cells (13).

Table1.9 Comparison of the evolutionary relationship between certain primate and nonprimate species (11)

Species	Index of Dissimilarity*
Primates	
Humans and apes	
Human	1.0
Chimpanzee	1.14
Gorilla	1.09
Orangutan	1.22
Gibbon	1.28-1.30
Old world monkeys	2.23-2.65
New world monkeys	2.7-5.0
Prosimians (e.g. lemur)	8.6-18
Nonprimates	
Bull	32
Pig	>35

*Based on reactivity in the microcomplement fixation test to human serum albumin. The greater the discrepancy from 1.0, the more distant is the evolutionary relationship with humans.

Table 1.10 Relative advantages and disadvantages of baboons and pigs as potential donors of organs and tissues for humans (13)

	BABOON	PIG
Availability	Limited	Unlimited
Breeding potential	Poor	Good
Period to reproductive maturity	3-5 years	4-8 months
Length of pregnancy	173-193 days	114 ± 2 days
Number of offspring	1-2	5-12
Growth	Slow (9 years to reach maximum size)	Rapid (adult human size within 6 months)**
Size of adult organs	Inadequate*	Adequate
Cost of maintenance	High	Significantly lower
Anatomical similarity to humans	Close	Moderately close
Physiological similarity to humans	Close	Moderately close
Relationship of immune system to humans	Close	Distant
Knowledge of tissue typing	Limited	Considerable (in selected herds)
Necessity for blood type compatibility with humans	Important	Probably unimportant
Experience with genetic engineering	None	Considerable
Risk of transfer of infection (xenozoonosis)	High	Low
Availability of specific pathogen-free animals	No	Yes
Public opinion	Mixed	More in favor

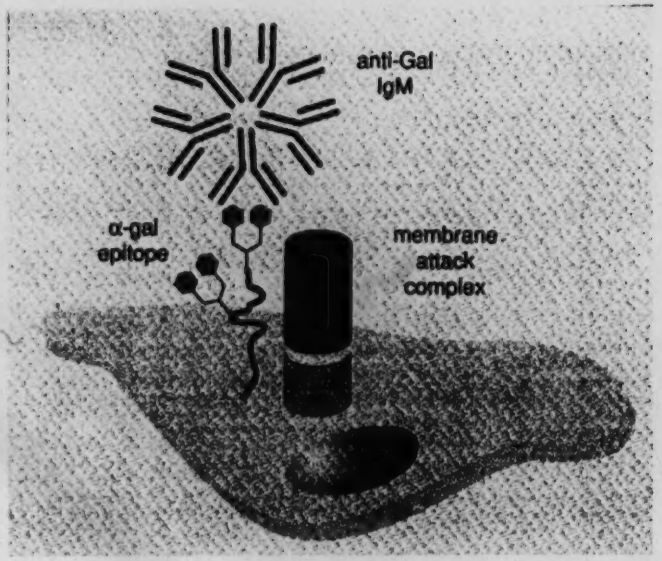
* The size of certain organs, e.g. the heart, would be inadequate for transplantation into adult humans.
** Breeds of miniature swine are approximately 50% of the weight of domestic pigs at birth and sexual maturity, and reach a maximum weight of approximately 30% of standard breeds.

Table1.11 Comparison of serum electrolyte levels (mMol/L) in humans and pigs (13)

Sodium	Human	140-160
	Pig	135-144
Potassium	Human	4.0-5.0
	Pig	3.6-4.8
Calcium	Human	2.2-2.5
	Pig	2.4-3.0
Phosphate	Human	5.0-8.3
	Pig	2.6-4.5
Chloride	Human	102-106
	Pig	97-108

Figure 1.2 Anti-α-gal destruction of xenograft cells. Anti-α-gal IgM is important in complement binding leading to cell death. Anti-α-gal IgG can also participate in antibody dependent cell toxicity.

Anti-Gal-Mediated Destruction of Xenograft Cells



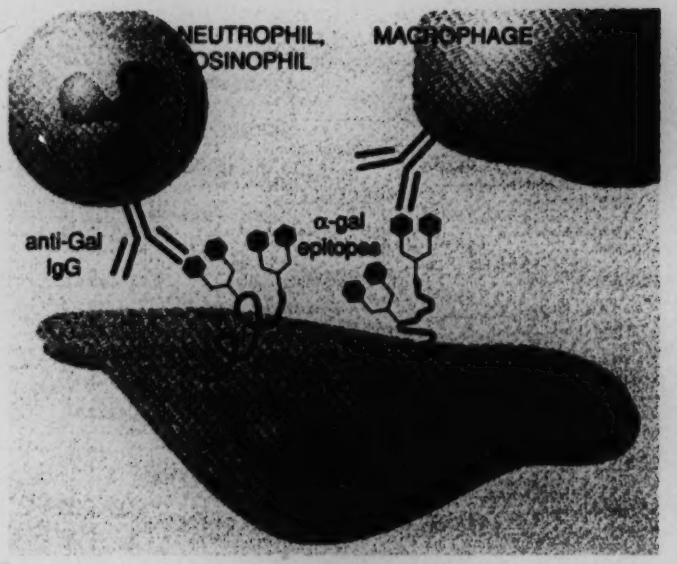


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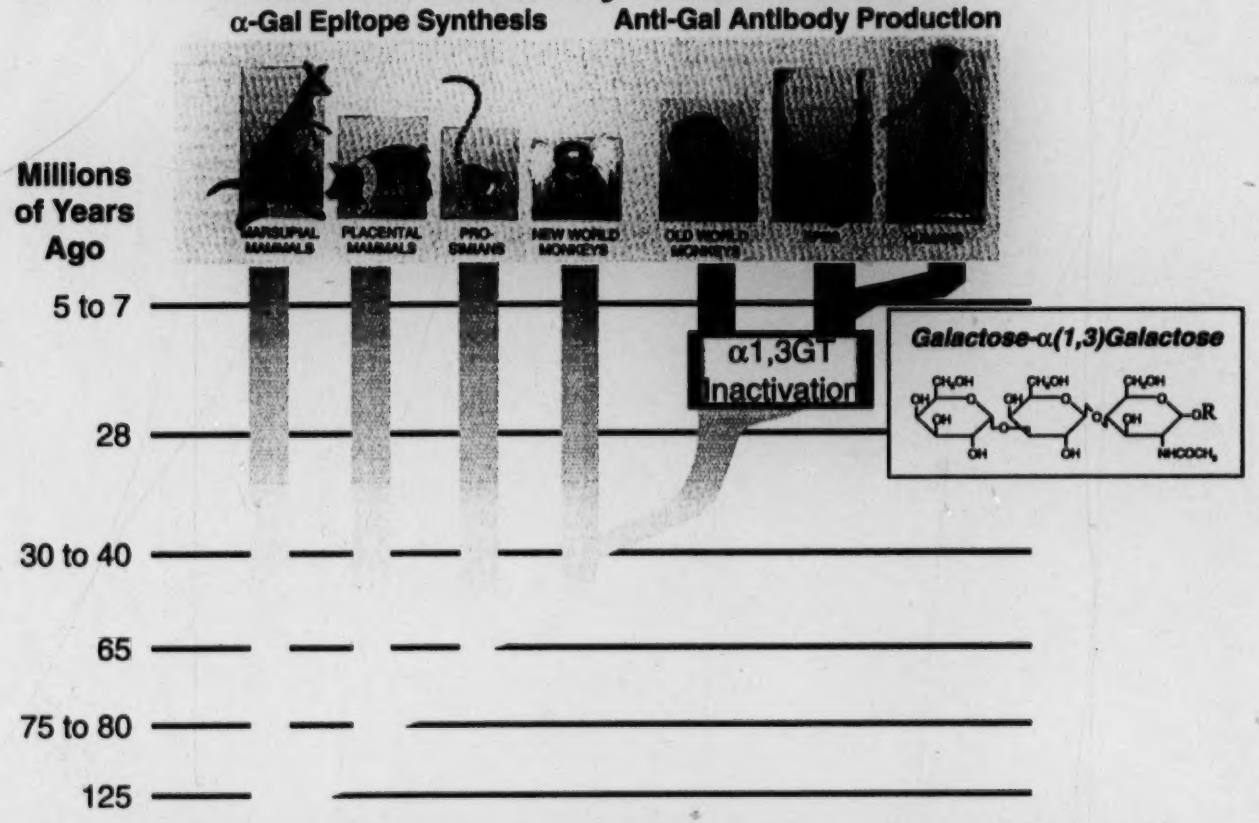
Figure 1.3 Reciprocal evolution of Galα(1,3)Gal epitopes and the anti-gal antibody in mammals. α1,3 galactosyltransferase enzyme inactivated approximately 30-40 million years ago (with the separation of new and old world monkeys). Humans, apes and old world monkeys do not express α-gal antigen and instead make anti-α-gal antibody. All other mammals express α-gal antigen.

Reciprocal Evolution of Galα(1,3)Gal Epitopes and the Anti-Gal Antibody in Mammals



Galili, Uri. Anti-Gal Antibody Prevents Xenotransplantation, *Science & Medicine*, Vol.5, No. 5 1998, p7

Table 1.12 Antibody and complement in donor-recipient combinations (11)

			Complement Cascade	
Recipient	**Donor**	**Graft Type**	**Alternative**	**Classical**
Human	Human (ABO compatible)	Allograft	no	no
Human	Human (ABO incompatible)	Allograft	no	yes
Human	Baboon Chimpanzee	Concordant xenograft	no	yes
Human	Pig	Discordant xenograft	no	yes
Rat	Guinea-pig	Discordant xenograft	yes	yes
Human	Rabbit	Discordant xenograft	yes	yes

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CHAPTER 2

Materials and Methods

Chapter 2: Materials and Methods

I. Experimental Animals

Outbred 5-6 kg, 14-21 day old Yorkshire/Landrace cross piglets were purchased from the University of Alberta Farms through Health Sciences Lab Animal Services at the University of Alberta. All animals received care in compliance with the guidelines governing handling of laboratory animals (Animal Ethics Committee, University of Alberta) in accordance with the Canadian Council on Animal Care Guidelines for the care and use of experimental animals.

II. Blood Products

A) Human

Human blood for all experiments was obtained from Canadian Blood Services (Edmonton, Alberta), from volunteer donors, and from patients undergoing therapeutic phlebotomies for hemachromatosis. It was confirmed with two hematologists an immunologist and other members of my supervisory committee that people with hemachromatosis are suitable for use in studies of xenotransplantation as the hemachromatosis has no bearing to the processes we were looking at (personal communication). The blood products obtained from Canadian Blood Services was either plasma or packed red blood cells (RBC). The blood obtained from volunteers or from people with hemachromatosis was anticoagulated with heparin, kept cold at all times (to preserve complement and antibody), and spun at 3000 rpm for 15 minutes at 4°C to

separate RBC from plasma. The plasma was frozen at < -20°C (usually -80°C) until used. The RBC were washed two times in 0.9% saline and then stored overnight (if needed) at 4°C in modified Kreb's-Henseleit buffer. Before use, the RBC were washed at least once more in fresh Kreb's-Henseleit buffer.

Blood collected was tested to ensure that the donor was essentially healthy (i.e. normal complete blood count, liver function, renal function, glucose, electrolytes, total antibodies, and complement). The blood was also typed (ABO Rh blood group typing) and the anti-A and anti-B antibody titers were determined via hemagglutination titers with commercially available RBC at the blood bank at the University of Alberta Hospital.

Human blood for use in Chapter 3 – development of a model of hyperacute rejection – was either individual people's blood or pools of 2-3 people from each blood group. Human serum (0.5 ml per person) for use in Chapter 4 – *in vitro* blood group studies – was again pooled by blood group to make the "*in vitro* pools". The number of individual donors per blood group pool were: 45 for blood group A, 18 for blood group B, 10 for blood group AB, and 45 for blood group O. Human plasma (250ml per person) for use in Chapter 4 – *ex-vivo* pig working heart blood group studies – was pooled by blood group to make the "*ex-vivo* pools". The number of individual donors per blood group pool were: 6 for blood group A, 5 for blood group B, 7 for blood group AB, and 6 for blood group O. Human whole blood and plasma for use in Chapter 5 were the same for Chapters 3 and 4 respectively.

B) Porcine

Autologous pig blood was used for all negative controls. To collect the blood, the following was done: the piglet was anesthetized, a tracheostomy was performed and the

piglet was put on a ventilator (see later). Having proper anaesthetic control of the piglet, a midline laparotomy was performed and the aorta and vena cava were dissected out. After performing the sternotomy and having the heart all prepared for cardioplegic arrest and explantation (see later), 3000 units of heparin (Leo Pharma Inc., Ajax, Ontario) was injected into the pulmonary artery to establish systemic anticoagulation. A few minutes later, a 16-gauge angiocath was introduced into the abdominal vena cava and this was attached to a 500mL bag of warmed modified Kreb's-Henseleit buffer to be used for volume resusitation if needed. A 14-gauge angiocath was introduced into the abdominal aorta to exanguinate the animal from the aorta into a 250 mL plastic bottle (Nalge Nunc International, Rochester, New York) via IV tubing (Baxter Corp., Toronto, Ontario). If whole blood was needed for the experiment, the piglet was volume resusitated through the vena cava towards the ending of the exanguination with the warm buffer until a total of about 350 mL of blood/buffer was collected from the aorta (this is the volume needed for the working heart apparatus to function). If only plasma was desired, then no volume resusitation was performed to avoid diluting the plasma. The collected porcine whole blood was spun at 3000 rpm for 10 min at 30°C by an assistant while the surgeon completed the heart harvest and hung the heart onto the perfusion rig. The plasma obtained after spinning the whole blood was usually ready for use in the heart (after a very brief warming to 37°C) by the time the heart was hung on the perfusion rig and homeostasis achieved.

III. Surgical Procedure

A) Anaesthetic

Ketamine was injected intramuscularly at a concentration of 100mg/kg and then ≤ 1% halothane with oxygen (3L per minute) via a face mask was used to achieve complete anaesthesia. The halothane was turned off within 5-10 minutes of starting the procedure. The piglet was kept well anesthetized and comfortable on just oxygen and the ketamine given earlier.

B) Tracheostomy and Ventilation

A tracheostomy was performed to allow proper oxygenation and ventilation which was needed once the thoracic cavity was opened. To perform the tracheostomy, a midline incision was made extending the entire length of the neck. The neck muscles were separated longitudinally allowing visualization of the trachea. A transverse incision was made in the trachea and a 5.0mm (internal diameter) endotracheal tube which had been cut so as to only be 5-6 cm long was inserted. After proper tying of the endotracheal tube in place, a ventilator (Fraser Harlake Anaesthesia ventilator, Model 701) was attached to the endotracheal tube. Parameters set on the ventilator were: respiratory rate of 15 breaths per minute, and minute ventilation rate of 2-3L per minute. The piglet was not ventilated at this time. Ventilation was only started once the chest was opened.

C) Laparotomy

If autologous pig blood was needed, then a laparotomy and pig blood collection as outlined in II(B) above was performed.

D) Sternotomy and Heart Harvest

The skin was incised from the suprasternal notch to the xyphoid process and the dissection carried down using electrocautery (Birtcher, Model 771, ElMonte, California) to the bone. The sternum was cut longitudinally using heavy scissors being careful not to injure the heart underneath, not to injure the brachiocephalic vein and not to go laterally and thereby potentially injure the internal mammary blood vessels. The chest incision was kept spread open with self-retaining retractors. The inferior vena cava was easily visualized and a 3-0 silk tie (Ethicon) was placed around it. The superior vena cava was then dissected out and a 3-0 silk tie placed around it as well. The thymic tissue on top of the heart was removed and the pericardium was opened and the majority of it cut out and discarded. The connective tissue between the proximal aorta and pulmonary artery trunk was separated to allow proper placement of the aortic cross clamp. Three thousand units of heparin (Leo Pharma Inc., Ajax, Ontario) were injected into the pulmonary artery to achieve systemic anticoagulation. If needed, the abdominal aortic exanguination was completed at this time (as outlined in II(B) above). The tie around the superior vena cava was then tied (the tie around the inferior vena cava was not tied allowing a vent for the heart). The inferior pulmonary vein on the right side was also cut to allow venting of the heart. The proximal thoracic aorta (aortic root), which had been separated from the pulmonary artery trunk, was then cross clamped using a vascular clamp and 10 mL of ice cold cardioplegia (30meq KCl, 2.5 g $MgSO_4$, 100ml THAM, and 22ml CPD in 250 mL

of D5W) was injected proximal to the aortic cross clamp to arrest the heart. Cold saline slush was then poured onto the heart to achieve topical cooling. The tie around the inferior vena cava was then tied and the heart excised out of the chest cutting the superior vena cava and inferior vena cava distal to the ties. The excised heart was then put into a cold saline slush bath while the superior vena cava was separated more fully from the aorta, aorta and pulmonary artery trunk were further separated, and the aortic root trimmed appropriately to be able to introduce onto the aortic cannula of the perfusion apparatus. The heart was briefly blotted between two gauze pads and weighed to determine the pre-perfusion weight of the heart. The heart was subsequently hung on the perfusion apparatus where langendorff perfusion with warm, oxygenated perfusate was started (see later). The entire ischemic time (from aortic cross clamp to time the heart was in langendorff perfusion) was on average 6-8 minutes.

IV. Isolated Heart Perfusion

A) Hardware

The hardware (**Figure 2.1**) consisted of water-jacketed perfusion glass chambers, low flow peristaltic pumps (Sarns Siok II Blood Pumps), hot water bath (42-45°C), in-line filters (40μM - Millipore), temperature probes (copper-constantan thermocouples type T with readers - Cole-Parmer, Model 08500-40), heart cannula, cannula holders, blood pressure monitor and transducer (Hewlett Packard T154B System), membrane oxygenator, defibrillator (Corbin-Farnsworth, Inc.), pacemaker (Medtronic 5375 Demand Pulse Generator, Minneapolis, Minnesota), air compliance chambers (20 cc syringe filled with 10 cc of air) and plastic tubing. The system was set up on a manifold to support two

experiments simultaneously by sharing carbogen (95% O2, 5% CO2) supply and warm water re-circulating throughout the jacketed glassware.

The water-jacketed perfusion chambers were made to specifications by the Technical Resource Group, Scientific Glassblowing Services, University of Alberta. The perfusing chambers have an inflow port, an outflow port to the heart and an overflow port (which was used to keep perfusate levels and thus load pressures on the heart at the desired levels). The cannulas for the heart were constructed in the lab out of metal cylinders (for the cannulas) and plastic for the cannula holders. Each rig had two cannulas (one for the aorta and the other for a pulmonary vein going into the left atrium). The internal diameter of the cannulas was 5mm and each had a lip of 1-2mm to allow the cannulated tissue to be tied to the cannula securely to prevent it from slipping off. The defibrillator was used at a setting of 75mV for 1 second using small paddles placed directly onto the heart. If pacing was required, the pacing wires were placed into the heart in different places depending on the function of the heart. If the atrial rate was too slow, the pacing wires were placed into the atria. If this did not increase ventricular rate or if there was evidence of complete heart block, then the ventricles were paced directly placing the pacing wires directly into the ventricles. The plastic tubing used in all places had an internal diameter of 8 mm.

The langendorff height was set at 70cm above the heart. The preload level was set at 11.5 mmHg and the afterload was set at 50mmHg. The oxygenation of the perfusate was maintained to keep a PaO_2 greater than 200mmHg and the temperature of the perfusate was kept between 39-40°C which is physiologic for a pig.

B) Perfusion buffer

A modified Kreb's-Henseleit buffer was used which consisted of the following (final concentration present in perfusate):

NaCl – 100 mM	KH_2PO_4 – 3.0 mM
KCl – 0 mM	$NaHCO_3$ – 25mM
$CaCl_2 \cdot 2H_2O$ – 3.0 mM	EDTA – 0.5 mM
$MgSO_4 \cdot 7H_20$ – 1.2 mM	glucose – 11 mM

All products except for EDTA were purchased from BDH Inc., Toronto, Ontario. EDTA was purchased from Sigma-Aldrich, Oakville, Ontario.

C) Langendorff mode

Langendorff perfusion is retrograde, non-working mode perfusion. In this mode of perfusion (**Figure 2.1(a)**), perfusate flows through the coronary arteries thus warming the heart and delivering oxygenated, nutrient rich perfusate causing the heart to beat. The heart is beating empty; it is not pumping blood against any resistance and thus is not generating work. The metabolic demands of a non-working (Langendorff) heart preparation is less than half that of a working heart preparation. However, before establishing a working heart preparation, one must always start off in Langendorff mode to allow the heart to recover from the cold ischemic arrest it has just been in.

The perfusion system was primed with 350mL of warm perfusate. Once the heart had been prepared (as outlined in III(D) above), the aortic root was cannulated with the perfusate running out of the aortic cannula to avoid getting any air into the coronary circulation. Care was taken not to cannulate the aorta too deeply as this would block flow to the coronary ostia as well as cause aortic valve incompetence. A bulldog clamp

was placed on the aorta to hold it to the cannula while a #1 silk tie (Ethicon) was used to secure the aortic root to the cannula. If the ventricles began to fibrillate (as occurred at this point not uncommonly), the heart was defibrillated (often only needing one shock) into normal sinus rhythm. The left inferior pulmonary vein was then identified and the left atrial cannula (attached to the preload chamber) was placed into this, bulldog clamped and tied with a #1 silk tie (similar in procedure to the aortic root cannulation). One needed to be careful not to cannulate the left atrium too deeply as this would lead to mitral valve dysfunction when the heart was placed into working mode. Perfusate was allowed to flow out of the preload chamber into the left atrium for a short period of time to "de-air" the left atrium through the other three pulmonary veins that were not tied off yet. While this "de-airing" was occurring, the three pulmonary veins were identified, bulldog clamped altogether and then a #1 silk tie was placed around them to seal off all outflow out of the left side of the heart except for the aorta. Care was taken when doing this last tie not to accidentially include the superior vena cava or the right atrium (and especially the sinoatrial node in the right atrium) into the tie as this would cause rhythm disturbances in the heart. The heart was allowed to retrograde (Langendorff) perfuse for approximately 15-20 minutes until homeostasis was established and the heart was functioning well. During this Langendorff time, samples were drawn and tested via a Stat-9 Nova Analyzer machine (Nova Biomedical, Boston, MA) to ensure oxygenation, pH, potassium and calcium were at desired levels. After langendorff perfusion for about 20 minutes, the working mode was established.

D) Working mode

Working mode perfusion is shown in **Figure 2.1(b)**. Buffer (200-220mL) was removed from the system with the heart still in retrograde (Langendorff) perfusion mode. Warmed RBC (100 ml) was then added and the heart allowed to retrograde perfuse with this perfusate for about 1 minute. Warmed plasma (300mL), of whatever group was being tested, was then added to the perfusion system with the heart still in Langendorff mode and after about 30 seconds in this mode, the working mode was established.

To establish the working mode, the flow from the main reservoir to the langendorff chamber was clamped off and the flow to the preload chamber was opened. At the same time, the flow from the langendorff chamber to the heart was clamped off, and the clamp from the heart to the afterload column was opened. Perfusate now flowed from the preload chamber into the left atrium (via the cannulated pulmonary vein) into the left ventricle which then pumped the perfusate against the afterload resistance of 50 mm Hg. This perfusate then overflowed into the main cardiac chamber to then return to the peristalic pump to start the sequence again. The perfusate going through the coronary arteries would drain into the coronary sinus, then into the right atrium, followed by the right ventricle and then the coronary effluent would be pumped by the right ventricle out of the pulmonary artery trunk that was still left. Measuring this coronary effluent and the output from left ventricular contraction (aortic output) gave one the cardiac output. A blood pressure transducer was placed in line with the afterload column so that the pressures generated by the heart could be recorded (systolic blood pressure and diastolic

blood pressure). Heart rate could directly be counted. This was a physiological system where the heart worked against a resistance and the parameters of function (heart rate, blood pressure, cardiac output) could be easily collected. Function measurments were made 5 minutes after switching to working mode and every 15 minutes therafter. Samples were taken from the pulmonary artery (coronary effluent) at time 0 (1 minute after switching to working mode once plasma was in the system), and at time end (heart failure) for various assays. Hourly samples were also taken from the preload chamber (oxygenated perfusate before going into heart) and from the pulmonary artery (deoxygenated perfusate) for measuring pH, PaO_2, potassium, calcium, and hematocrit. These values were used to keep the environment as homeostatic as possible (e.g. adding $NaHCO_3$ if pH was too low). Glucose (100mg) and insulin (1 unit) were added every hour to ensure that metabollic substrate deficiency was not the cause of heart dysfunction.

After heart failure, the heart was removed from the perfusion chamber, drained of any perfusate and weighed. Heart function was calculated by the formula (cardiac output x peak (systolic) blood pressure x heart rate)/1000. Heart failure was determined when the above equation had a value less than 10 (See discussion section of Chapter 3 for explanation of the number chosen.).

When perfusing hearts with just plasma, it was found that if one followed the protocol outlined above (i.e. remove buffer and then add plasma in Langendorff mode before switching to working mode), there were some plasma samples (eg. A and O) where the hearts would rapidly be failing in Langendorff mode and one could not successfully convert to working mode, or if one could convert to working mode, the hearts rejected too quickly to be able to measure working mode parameters. At times, one

did not have enough time to measure time zero pH, Ca^{2+}, and other parameters to ensure all was well before the heart died. One also could not be sure if the heart failed due to technical problems (which did occur occasionally) or if it was rejection since failure occurred very quickly. To address these problems, the technique was modified as follows. After Langendorff perfusion with buffer, one switched to working mode as described earlier with buffer and let the heart function in working mode for 10 minutes with buffer. Plasma was subsequently added while the heart was in working mode and time 0 began from there. This method enabled working mode measuremnts to be made for all plasma hearts and be confident that heart failure was not secondary to a technical problem. This method was compared to the method described earlier (whole blood) and similar results were found – thus, the 10 minutes of buffer working mode were non-consequential. Hearts done by either technique could be compared.

V. Pathology/Immunofluorescence

After heart perfusion, hearts were removed from the perfusion circuit. Five pieces – one each from the anterior left ventricle, anterior right ventricle, posterior heart, interior interventricular septum, interior heart (usually papillary muscle) – were taken for pathologic examination. The pieces were cut into ~ 1cm x 1cm cubes and put into 2.5% formaldehyde for Hematoxylin and Eosin staining or they were cut into ~ 2mm x 2mm sections and put into MMD media at 4°C for immunofluorescence. The pathology samples were taken to the University of Alberta Hospital Pathology Department where they were processed.

The formaldehyde samples were embedded in paraffin blocks, cut and put onto slides. They were stained with hematoxylin and eosin and graded by a blinded pathology

fellow on a scale of 0 to 4 for evidence of hyperacute rejection (hemorrhage and thrombosis, 0 being <25% of section, 1 being 25<50% of section, 2 being 50<75% of section, 3 being 75<100% of section, and 4 being 100% of section).

The MMD samples were washed in MMD buffer and then frozen at -70°C in OCT. They were later microtomed into 6μm thick sections at -20°C, put onto slides and left to dry overnight. The samples were then washed in acetone, and then PBS before immunofluorescence staining. There were usually 5 heart sections per slide with one of these sections serving as the negative control for that stain.

All antibodies used for immunofluorescence were tested against known standard postive controls that were used by the hospital for clinical patient samples. Antibodies were rabbit-anti-human IgM, rabbit anti-human IgG, rabbit anti-human C3, and rabbit anti-human C1q. All antibodies used for immunofluorescence were FITC conjugated and were purchased from Dako. For immunofluorescence staining, the negative controls were blocked with unlabelled rabbit-anti-human antibody (IgM, IgG, C3, C1q as appropriate) for 20 minutes at room temperature in a humidity box. As this antibody was the same as the FITC conjugated antibody, but without the FITC, it bound to any antibody on the sample from the heart perfusion and thus prevented the FITC conjugated antibody from binding. The excess antibody was washed off. FITC-conjugated antibody was subsequently added to all 5 sections on the slide and incubated for 30 minutes at room temperature in a humidity box. The antibody was then washed away using PBS. This washing step was repeated 3 times. A coverslip was applied using a mounting media specific for immunofluorescence and the slides stored at 4°C until read. The slides were read by a pathology fellow blind to the identities of the sections. The sections were

scored on a scale from 0 to 4 depending on the amount of staining (scale as for hemorrhage and thrombosis above).

VI. Total IgG/IgM/IgA, CH50, CK measurements

If whole blood was being used, then after sample collection the blood was immediately spun at 1800rpm x 15 min at 4°C and the supernatant (plasma) was collected. For all samples (except CH50 complement samples), the sample was kept at 4°C. All CH50 samples were frozen at < -20°C (usually – 80°C). All samples were taken to the University of Alberta Hospital Department of Lab medicine to be analyzed. If just plasma was being used, the same procedure was followed minus centrifuging.

A) Total IgM, IgG, IgA measurements

The Department of Laboratory Medicine at the University of Alberta Hospital performed the measurments. The principle for measurments were changes in turbidity. All kits were purchased from Roche and all measurments were done on a Hitachi 917 spectrophotometer. Briefly, for each sample, an absorbance (turbidity) was measured at one wavelength. An "anti-antibody" was then added and the change in absorbance measured. Using a standard curve, one was able to determine the concentration of the antibody of interest in the sample. The wavelengths used for the measurements were as follows: IgM (700nm and 340 nm), IgG (600nm), IgA (800 and 600 nm).

B) Creatine Kinase (CK) Measurement

Plasma samples were sent to the University of Alberta Hospital Department of Laboratory Medicine where CK measurements were made using a modification of the UV enzymatic determination. The assay kits were purchased from Roche and the

measurements were done using the Hitachi 917 machine. The principle of the assay was that a change in absorbance at 340nm due to formation of NADPH was directly proportional to the CK activity

C) Total Complement (CH50) Activity

CH50 assays were done using the Cobas Miras kit which facilitates a color change which is proportional to the complement activity of the sample. Absorbance changes are read with the Hitachi 917 spectrophotometer at a wavelength of 340nm.

D) Anti-A and Anti-B Titres

Plasma samples were sent to the University of Alberta Hospital blood bank for determination of anti-A and anti-B isohemagglutinin titres. Simple hemagglutination assays were performed in which the sample plasma (of a known blood group) was diluted to at least 1:512 and then the specific red cell (A_1 and B purchased from Dominion Biologicals) suspension (2-4%) was added and the mixture incubated at 4°C overnight. Hemagglutination was read with the naked eye. The titre was stated to be the point where there was still 1^+ hemagglutination. Appropriate controls were used.

VII. Isolation and Culture of Pig Aortic Endothelial Cells (PAEC)

Thoracic and abdominal aortas were harvested from 5-6kg piglets or occasionally from 40-50kg pigs under sterile conditions and put into 50ml conical tubes (Falcon) with DMEM tissue culture media (Gibco BRL Life Technologies, St. Burlington, Ontario) for transport to the tissue culture hood. The aortas were put into a petri dish with media and

cut longitudinally to expose the inner lumen. Blood was removed by gentle irrigation with tissue culture media and then aortas were transferred to a new petri dish with fresh media. The endothelial cells were removed from the aortas by very gentle scraping using a cell scraper (Fischer Scientific, Whitby, Ontario) with a single pass only (to avoid contamination with fibroblasts or smooth muscle cells), and then collected and pipetted into a 50 ml conical tube and spun at 1200rpm for 10min at 30°C. The supernatant was aspirated and the cell pellet resuspended in fresh media and respun. This washing step was repeated once more. After the last wash, the cells were resuspended in 7.5ml of complete media, DMEM with 10% FCS (Hyclone Laboratories, Logan, VT) plus 1% penicillin/streptomycin (Bio Whittaker, Walkersville, Maryland), 2mM glutamine (Gibco BRL-Life Technologies, St. Burlington, Ontario), and 1mM Na pyruvate (Sigma-Aldrich, Oakville, Ontario) and pipetted into a T25 flask (Fischer Scientific) which had been precoated with sterile 0.1% gelatin. The cells were incubated at 37°C, 5% CO_2 overnight. Usually by this time, some endothelial cells had attached to the flask and there were a large number of floating RBC. The media was gently aspirated out of the flask and fresh media (~5ml) was added to the flask and rinsed over the bottom of the flask. This was aspirated and discarded. The process was repeated another two times. At this point, examination revealed endothelial cells attached to the bottom of the flask with minimal RBC contamination. About 7.5ml of fresh complete media was added and the flask incubated at 37°C, 5% CO_2 for another 24 hours when the same procedure was performed again – this time to eliminate any contaminants that may be present. Endothelial cells were identified by morphology. Media changes were done every 2-3 days subsequently. Media changes consisted of pipetting out ~80% of the media and replacing it with the

equivalent amount of fresh complete media. When the cells were >75% confluent, the cells were split. To split the cells the bottom of the flasks were scraped and the resultant cell suspension pipetted into one or more 50ml tubes to which was added an equivalent volume of fresh complete media. The cell suspension was spun at 1200rpm for 10min at 30°C, supernatant aspirated and cell pellet resuspended in fresh complete media. One T25 flask which was >75% confluent was transferred into one T75 flask which again had been precoated with 0.1% gelatin. Media was changed every 2-3 days. Once a T75 flask was >75% confluent, the cells were split 1:3 (one T75 flask into three T75 flasks). Cells of passages 3 to 10 were used for experiments. With the above technique, we were able to obtain >90% pure endothelial cells (**Figure 2.2**).

VIII. ELISAs

A) Anti-α-Gal ELISA

90-BSA which is the α-gal trisaccharide antigen (galactose α 1,3 galactose β1,4 glucose) covalently bound to bovine serum albumin was obtained from the Alberta Research Council (Edmonton, Canada). Ninety-six flat-bottomed well ELISA plates (Falcon Probind 3915, Becton Dickinson, Pont De Claux, France) were coated with 0.5μg/well of 90-BSA in 50 mM NaHCO3 (pH=9.55) buffer and incubated overnight at room temperature in a humidity box. The plates were covered with parafilm and outer perimeter wells were not used due to the risk of evaporation. The next morning the coating buffer was discarded and the plates were washed four times with wash buffer (TBS/0.1% Tween) leaving the last two washes in for ~5 minutes each. The plates were then blocked with PBS/2% BSA solution (150 μl/well) for 2 hours at room temperature. The block solution was removed and 100μl/well of sample (primary antibody) added to

the wells. Sample dilutions were prepared in PBS/1%BSA. For anti-α-gal IgM ELISAs, most samples were diluted 1/20 and for anti-α-gal IgG ELISAs, most samples were diluted 1/50. The plates were incubated at 37°C for 1 hour followed by 3-4 washes as described earlier. Secondary antibodies goat anti-human IgM-horseradish peroxidase (HRP) (Cedarlane Labs, Hornsby, Ontario) used at 1:1000 dilution or goat anti-human IgG-HRP (Cedarlane Labs) used at 1:1000 dilution were prepared in TBS/1% BSA. The secondary antibody was added at 50μl/well and incubated at 37°C for 1 hour afterwhich the plates were washed 3 times. The chromogen solution was made as follows: 7.5ml of 0.05M citric acid, 7.5ml of 0.1N $Na_2HPO_4 \cdot 7H_2O$, and 7.5mg OPD (Sigma-Aldrich) per 96 well plate. Just prior to addition to the plates, 12μl of 30% H_2O_2 (Sigma-Aldrich) was added to the solution. The solution was well mixed and 100μl/well was added to all the wells. The plates were incubated in the dark at room temperature for 45 minutes. At this time, the optical densities were read on an ELISA plate reader with test wavelength of 490nm and reference wavelength of 600nm.

As there was no purified anti-α-gal antibody of known concentration available to be able to use for a standard curve, and as it was not possible to do all ELISAs on the same day on one plate, reference serum was used on each plate. The optical density of this reference serum was then divided into the optical densities of the test sera to arrive at a standardized number. This standardized number was used in the results and allowed one to compare ELISAs done on different plates at different times.

B) Anti-PAEC ELISA

Anti-PAEC ELISAs were done exactly the same as anti-α-Gal ELISAs except for the few steps mentioned below.

PAEC cells grown in T75 flasks were split as described earlier and plated into 96 well flat bottom plates (Falcon) which had been precoated with 0.1% gelatin. When cells were ready, media was aspirated out and the cells were fixed with PBS buffered glutaraldehyde for 5 minutes at room temperature. After fixation, the cells were washed 4 times with wash buffer. The plates were then blocked with PBS/2% BSA and the rest of the steps were as described for the anti-α-gal ELISA.

IX. MTT Cytotoxicity Assay

The procedure followed was similar to Mosmann *et al.* (1). PAEC were plated out into 96 well flat bottom plates and used when confluent as described under anti-PAEC ELISA above.

Media was removed from the wells and warm M199 media, without phenol red, (Gibco BRL-LifeTechnologies) was added to all wells (100µl/well) to wash the cells of any existing complete tissue culture media. Sample plasma was then diluted (if needed, in M199 media) and added at 100µl/well to appropriate wells. M199 at 100µl/well was added to the outermost wells of the plate (which had no cells). Positive control serum (human plasma with high cytotoxicity) was added at 100 µl/well to the appropriate wells. The negative control wells received just M199 media. A few wells received 100µl/well of 0.5% SDS in M199 media as a further positive control. A reference standard serum was added to all plates to allow comparison between plates.

The plates were incubated at 37°C for 1.5 hours after which the contents of the various wells were aspirated out in appropriate fashion ensuring no cross contamination into other wells. Standardized rabbit complement (Cedarlane Labs) was diluted in M199 media to a final concentration of 25% of the stock dose in the vial. 100µl/well of this

complement was added to all wells including the negative control wells. The plates were incubated at 37°C for 30 minutes. Complement was aspirated and 150µl/well of M199 added to all wells and subsequently aspirated as a wash. The wash was repeated twice more.

MTT (3-4,5-dimethyldiazol-2-yl-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich) is a soluble yellow reagent that is taken up by living cells and is metabolized in the mitochondria to produce violet crystals. These crystals can be solubilized by isopropyl alcohol and the resultant optical density of the color measured. The higher the optical density, the more living cells present. MTT was diluted in M199 media to a final concentration of 1mg/ml. 50 µl/well of this solution was added to all wells. The plates were incubated for 3 hours at 37°C at which time, violet crystals were observable in the negative controls with light microscopy. The MTT solution was aspirated and 100 µl/well of isoproyl alcohol (Sigma-Aldrich) was added and mixed well with the cells to lyse the cells and dissolve the crystals. The plates were then read on an ELISA plate reader at a test wavelength of 530nm and a reference wavelength 750nm. The optical density of the samples and reference standards was compared to the positive (all dead) control to determine percent survival. This percent survival was then normalized by the percent survival of the reference standard to come up with a standardized survival value. This value was used to compare plates and do statistical analyses.

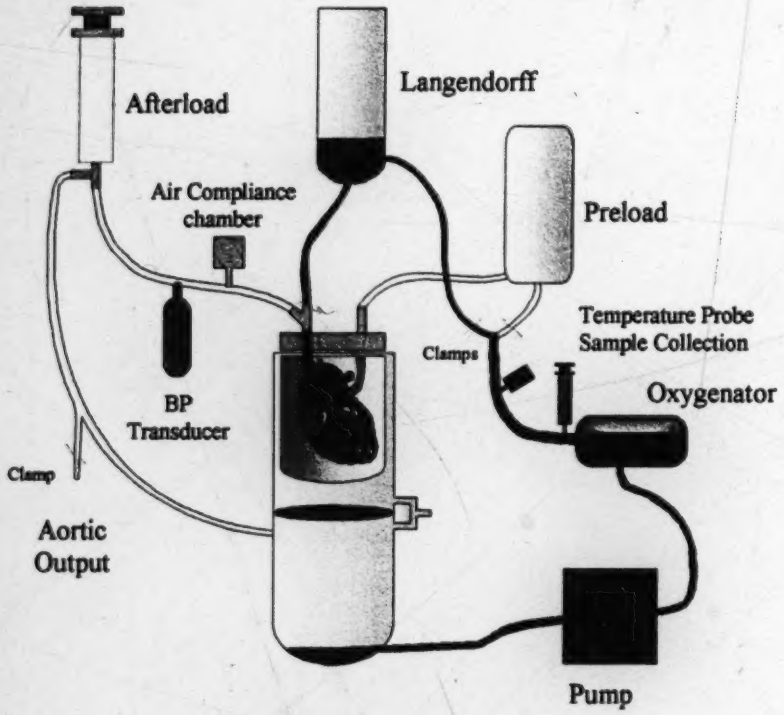
X. Immunoadsorption

Blank immunoadsorbant (processed Chromosorb P) and anti-α-gal immunoadsorbant (S90/IB6) were purchased from the Alberta Research Council (Edmonton, Canada). Immunoadsorption was acheived using 0.2g of immunoadsorbant

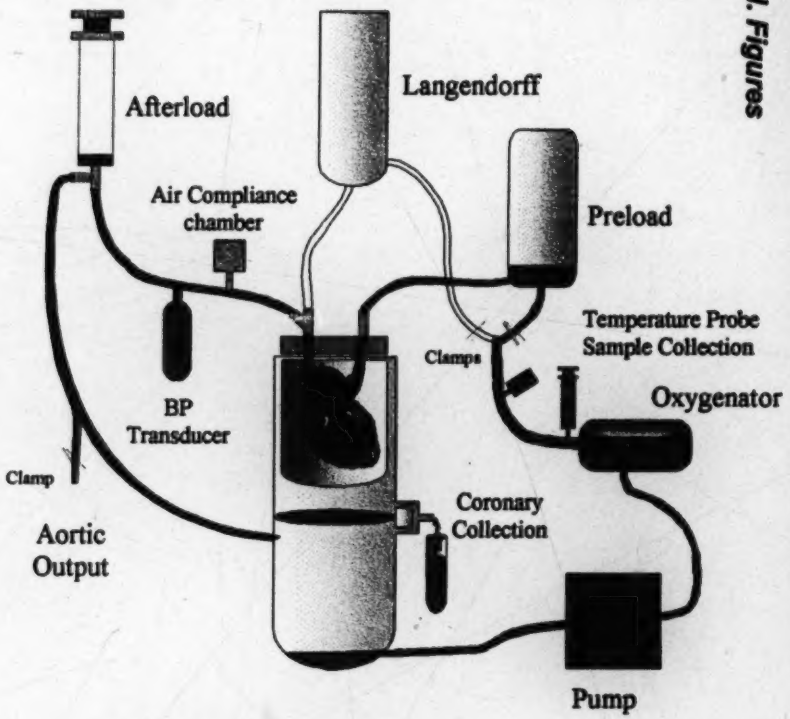
per ml of plasma rotated overnight at 4°C in 50 ml conical tubes. After the adsorption, the tubes were centrifuged at 1800rpm for 5 min at 4°C and the supernatant collected. The supernatant was left at 4°C until used. Pre-adsorption and post-adsorption samples were taken for various assays.

XI. Statistical Analyses

Statistical analyses were done using SPSS version 10.0. Data were examined for normality of distribution. If the data were normally distributed (which included all data except for creatine kinase change and weight change measurements), t-tests and analyses of variance with post hoc Tukey tests were done where appropriate. For data that were not normally distributed, non-parametric statistical tests (Mann-Whitney-U or Kruskal-Wallis as appropriate) were performed. Univariate and multivariate linear regression was also performed to determine predictors of survival in some of the sections. For all statistical tests, a value of $p<0.05$ was considered statistically significant. Data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated.



(a) Langendorff mode perfusion



(b) Working mode perfusion

Figure 2.1 **(a)** Langendorff mode perfusion – warm perfusate is pumped through the oxygenator into the Langendorff chamber which then perfuses the heart via gravity. In Langendorff mode, the heart beats empty. **(b)** Working mode perfusion – warm perfusate is pumped through the oxygenator into the preload chamber which empties into the left atrium. The perfusate then goes into the left ventricle and now the heart has to pump this perfusate up against the afterload resistance. The heart has to work, generating mechanical force. One can measure the blood pressure, the cardiac output (aortic output + coronary output) and the heart rate. Coronary collections can be analyzed for various factors such as antibodies.

Figure 2.2. Representative photomicrograph of pig aortic endothelial cells used for various assays. Magnification 10x.

XIII. References

1. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotxicity assays. *J Immunol Methods* 1983; 65: 55.

CHAPTER 3

Methodological Considerations in the Development of a Hyperacute Rejection Porcine-to-Human Working Heart Xenotransplantation Model

Chapter 3: Methodological Considerations in the Development of a Hyperacute Rejection Porcine-to-Human Working Heart Xenotransplantation Model

I. Introduction

The initial problem in transplantation of a pig organ into a human is hyperacute rejection (1). Hyperacute rejection involves many factors including components of the immune, coagulation and hematological systems (2). There are many model systems one can use to study hyperacute rejection. These include *in vitro* systems, *in vivo* systems and *ex-vivo* systems. Each system has its advantages and disadvantages. An *ex-vivo* system allows one a sensitive way to examine organ function, allows one the ability to test various factors independently or in different combinations, allows one more control over different variables and allows one to have many replicates and test many different factors - all relatively inexpensively and practically. An *ex-vivo* porcine working heart model needed to be established to investigate various factors related to hyperacute rejection in pig to human cardiac xenotransplantation.

II. Objectives

1. To develop and optimize an *ex-vivo* porcine working heart model.
2. To further develop and validate the above model as a model of hyperacute rejection (pig to human combination).
3. To investigate the importance of various factors in causing hyperacute rejection in the pig to human combination.

For objective number three, we hypothesized that anti-α-gal antibodies, anti-pig aortic endothelial cell (PAEC) antibodies, and complement would be important factors in causing hyperacute rejection.

III. Methods

Please refer to Chapter 2 of this thesis

IV. Results

A) Developing and Optimizing an *Ex-vivo* Porcine Working Heart Model

Figure 3.1 shows survival of working hearts based on calcium concentrations. A calcium concentration of 3.0mmol/L is best and this was used for all of our perfusions.

Figure 3.2 demonstrates the survivals obtainable with various perfusates. If buffer is used, average survival is about 180 minutes; if autologous pig plasma is used, survival is about 210 minutes and if pig whole blood is used, the survival time is about 345 minutes. The addition of pig red blood cells significantly improves survival time. This graph demonstrates the average limits of detection for our system depending on the perfusate used.

B) Determining if We Have a Model of Hyperacute Rejection

Figure 3.3 shows that when using whole blood, autologous pig blood (negative controls, n=10) survived 345±17 minutes versus 169±23 minutes for human blood (positive controls, n=27) ($p<0.001$, t-test). The average 180 minute function (180fxn) for the negative controls is also significantly ($p=0.006$) better than the positive controls (**Figure 3.4**). Weight change, a measure of edema accumulation, (**Figure 3.5**) was

0.03±0.01 g/min in the negative controls versus 0.32±0.12 g/min in the positive controls ($p<0.001$, Mann-Whitney U test). The creatinine kinase (CK) change, a measure of the amount of heart muscle damage, **(Figure 3.6)** was 21±4 U/L/min in the negative controls (n=7) versus 78±29 U/L/min in the positive controls (n=22) giving a p-value of 0.110 (Mann Whitney U). Light microscopy of the negative controls **(Figure 3.7(a))** shows minimal hemorrhage and thrombosis. However, light microscopy of the positive controls **(Figure 3.7(b))** shows severe hemorrhage and thrombosis consistent with hyperacute rejection. Immunofluorescence of the negative controls **(Figure 3.8(a-c))** shows no human IgM/ IgG binding, no C1q complement (measure of classical complement activity) binding compared to the positive controls **(Figure 3.8(d-f))** which show comparatively more binding of all of these factors. These findings are consistent with hyperacute rejection. **Figures 3.9 and 3.10** show the pathology scores assigned to the various hearts for the various factors comparing the negative and positive controls. Positive control hearts consistently scored higher than negative control hearts, except for C3 binding (not shown).

To ensure that the differences observed between the positive and negative controls were not related to some technical bias, the technical factors were reviewed. **Table 3.1** is a univariate linear regression analysis of the negative control hearts showing that no one factor has a greater effect than the others on heart survival or function. **Table 3.2** demonstrates that if technical issues played a role, then the negative control group should have done worse, since total OR time, Hct, and K^+ were all worse in negative controls. From the above results, it was apparent that the model was a model of

hyperacute rejection and that one could detect differences that needed to be detected (except for CK changes and C3 complement binding).

C) Determining the Role of Human Red Blood Cells

While validating the model, considerable variability in the positive controls was observed with a few hearts surviving and functioning just as well as the negative control hearts. To investigate this the role of human red blood cells (hRBC) was examined. **Figure 3.11** shows pig hearts perfused with buffer compared to hearts perfused with different washed hRBC (no human plasma, hematocrit ~15%). The average survival of the hRBC group was 143 ± 51 minutes (n=6). It appears that hRBC have a variable effect on heart survival. Three of these 6 hearts had severe hemorrhage and thrombosis (looking like hyperacute rejection) when examined grossly even though there was no plasma present in the perfusate (data not shown). Immunofluorescence of these hearts revealed no human antibody staining (data not shown).

Because of the variability present with hRBC, and because factors important for rejection (antibodies and complement) are present in plasma, positive and negative control hearts were done using just plasma to ensure that a model of hyperacute rejection did exist. Hearts perfused with autologous pig plasma (n=2) survived 211±15 minutes which was significantly ($p<0.001$ t-test) greater than hearts perfused with human plasma (n=13) 71±31 minutes ensuring that the model was a functional model of hyperacute rejection. Technical factors were biologically similar for these two groups. **Figure 3.12** compares hearts perfused with plasma only to hearts perfused with the the exact same plasma and RBC. Again variability is seen with the whole blood groups; however, in

general, hearts perfused with whole blood tend to survive longer. **Figures 3.13 and 3.14** compare all the hearts perfused with just plasma (n=13) to hearts perfused with human whole blood (n=27). **Figure 3.13** shows that hearts perfused with human whole blood survived significantly longer; 169±23 minutes versus 71±31 for hearts perfused with plasma alone ($p=0.017$ t-test). **Figure 3.14** shows that these hearts also functioned significantly better ($p<0.001$) than hearts perfused with human plasma alone. Technical perfusion conditions were similar for the groups except hematocrit and PO_2 which were higher in the whole blood group. Thus, it appeared that in general, having hRBC (using whole blood) was beneficial. However, it had still been observed that some hearts had performed poorly with whole blood. It was hypothesized that the type of RBC made a difference. **Figure 3.15** shows that survival in the group receiving human O RBC (universal donor), regardless of human plasma blood group, was 226±25 minutes versus 71±23 minutes in the group receiving non-O (i.e. A, B, and AB) RBC ($p<0.001$, t-test). Function (**Figure 3.16**) in the two groups however, was statistically similar ($p=0.148$) suggesting that RBC in general do give similar assistance with function but that human O RBC tend to have a protective effect for survival. **Table 3.3** shows that technical factors were biologically similar between the O RBC group and the non-O RBC group, thus differences in technique were not the reason for the differences seen. O RBC however were not protective enough to prevent hyperacute rejection as the O red blood cell group still had significantly ($p=0.04$, t-test) shorter survival times (226±25 minutes vs. 345±17 minutes) compared to the pig whole blood negative control group (**Figure 3.17**).

D) Determining Important Predictors of Hyperacute Rejection

Discovering that one factor (O red blood cells) had such an impact on heart survival, the data was examined to determine other predictors of rejection of porcine working hearts perfused with human blood. **Tables 3.4(a-d)** show the results of the various linear regression analyses. It was confirmed that the variables tested had a linear or near linear distribution with survival and function before doing regression analyses on them. In the univariate analysis using all positive control whole blood hearts, the significant predictors for survival included the total IgM level and the anti-α-gal IgM level. For function, the important variables in the multivariate analyses were total IgM, anti-α-gal IgM and anti-PAEC IgM **(Table 3.4(a))**. When these significant variables were put into a multivariate analysis, the only significant predictor for both survival and function was the anti-α-gal IgM level **(Table 3.4(b))**. As one may expect differences based on RBC type, the same regressions were done on O RBC hearts and non-O RBC hearts. In the hearts perfused with whole blood having only O red blood cells, there were no statistically significant variables in the regression analysis **(Table 3.4(c))**. In hearts perfused with non-O red blood cells, only the anti-α-gal IgG level was significant **(Table 3.4 (d))**. Thus, it appeared that the anti-α-gal antibody levels alone (IgM and IgG) were important predictors of rejection in the model (not total antibody level, or anti-pig aortic endothelial cell antibody levels, etc.).

Having learned the importance of the anti-α-gal IgM and IgG levels in predicting rejection, it was confirmed that these levels in the plasma only group and the whole blood group **(Figures 3.13 and 3.14)** were similar **(Table 3.5)**. Thus, there was a beneficial

effect from RBC and not just different levels of anti-α-gal IgM/IgG explaining the findings. Secondly, it was confirmed that the anti-α-gal IgM/IgG levels in the O RBC group and the non-O RBC (**Figures 3.15 and 3.16**) group were similar (**Table 3.6**). Thus, the benefit offered by O RBC was not due to different anti-α-gal IgM/IgG levels.

Lastly, the minimum amount of anti-α-gal IgM required to cause hyperacute rejection was determined. Blood group AB plasma was used to avoid any possible interactions that the anti-A and anti-B antibodies may have in the other blood groups. Doing a titration (serial dilution) as demonstrated in **Figure 3.18**, it was determined that the minimal amount of anti-α-gal IgM needed to start rejection is 7.5 mg/L. The calculations will be described in the discussion.

V. Discussion

A) Development and Optimization of an *Ex-vivo* Porcine Working Heart Model

i) Important Operative Issues

For anaesthesia, using high doses (100 mg/kg) of ketamine IM with very small amounts of inhaled halothane (≤ 1%) was best. Initial trials with higher doses of halothane (5%) for induction followed by 1-2 % for maintenance often gave poor heart contractility and bradycardia occassionally leading to asystole. With our technique, the piglets had good analgesia, and anaesthesia, and it was possible to turn the halothane off within 5-10 minutes of starting the operation (which usually took about 30 minutes to complete).

Pigs that were 5-6kg in weight usually had hearts 25-35g in weight and this was ideal for our system. Pigs smaller than 3kg had aortas that were too small for the aortic cannula (leading to aortic valve incompetence) and pigs larger than 7kg in size had hearts

too large to fit in the perfusion chamber once hyperacute rejection (with heart swelling) started. One could obtain ~150-200mL of whole pig blood from the 5-6 kg piglets by the bleeding technique described in the methods section. If volume resuscitation was used, a total of about 350-400mL of blood/buffer could be obtained (having a hematocrit of about 13%). The electrolyte composition of this blood/buffer mixture was acceptable with minimal hemolysis and a normal potassium level. Previous attempts to just suction blood out of the piglets' chest after heart explantation gave significant hemolysis and high potassium. Caution did need to be used during volume resuscitation as sometimes the hearts would get very distended and contractility as well as heart rate would decrease. This problem usually resolved on its own within a minute and the heart started beating well. Occassionally, slowing down the intravenous fluid resuscitation rate and temporary cardiac massage was required. The hematocrit of 13% was not a major problem as one still obtained good survival and function and an ability to detect differences between groups (as will be presented later). Ischemia times ranged from 6-8 minutes (with > 95% being cold ischemia time).

ii) Apparatus Related Issues

Using a membrane oxygenator proved to be very important in the model. Bubbling oxygen via thin cannulas into the various chambers did not provide sufficient oxygen. The oxygenation saturation of the blood going to the heart using this technique was only ~75% which is a PO_2 of about 45 mmHg (saturation of venous blood oxygen). Using the membrane oxygenator allowed one to get an oxygen saturation of 100% with a PO_2 over 400 mmHg. Bubbling oxygen into the various chambers also introduced air into the circuit which would often embolize into the heart leading to poor function.

Lastly, bubbling oxygen into the chambers would produce foam which lead to volume loss. Using a membrane oxygenator eliminated all of these problems.

iii) Buffer and Perfusion Related Issues:

When hanging the heart on the perfusion circuit, it was important firstly not too have the aortic cannula in too deep as this would obstruct coronary flow. In addition, it was important not to put the left atrial cannula too deep into the left atrium otherwise one could obstruct the mitral valve apparatus and cause cardiac dysfunction. Also, it was important to ensure that the cannulas had a lip on them so that this could be used to anchor the heart properly and avoid it sliding off the cannulas.

The buffer used had to be optimized to work in our system. During the long perfusion of the hearts, there is some hemolysis and potassium levels do rise. Hyperkalemia can lead to cardiac dysfunction and arrest. To avoid quickly rising potassium levels, we modified the Kreb's-Henseleit (KH) buffer by removing potassium chloride from the recipe. Thus the potassium level in the buffer was 3.0mmol/L compared to the 5.9mmol/L in the original KH recipe and this made an important difference. The KH recipe allows one to choose calcium concentrations between 1.0mmol/L to 3.0mmol/L. Pigs normally have higher calcium concentrations (see Chapter 1, **table 1.11**). **Figure 3.1** shows a small experiment done to pick the ideal calcium concentration (3.0mmol/L) to use for the studies. The advantage of having the higher calcium levels is that as hyperkalemia develops in the working hearts with hemolysis and time, the higher calcium levels can help stabilize the myocardial cell membrane to help decrease problems with arrhythmias. The potential disadvantage of having higher calcium levels is that calcium has second messenger roles and is involved

in coagulative necrosis. One may be concerned that this higher calcium concentration may cause problems or bias one group or the other. **Table 3.2** shows that calcium concentrations were similar between negative and positive controls. Thus calcium level should not be a problem. **Figure 3.2** demonstrated the average limits of perfusion with various perfusates. It was important to learn that an hematocrit as low as 13% could give prolonged survial (normal hematocrit is ~30-40%).

iv) Functional Measurements for Working Hearts

The measure of function used for this model was the product of cardiac output (CO) in mL/min, peak(systolic) blood pressure generated by the heart (SBP) in mmHg and heart rate (HR) in beats per minute (i.e. CO x SBP x HR). For practicality of graphing, the product (CO x SBP x HR) obtained was divided by 1000 and that number was plotted. The average function at 180 minutes was used as the measure of function to make number handling and statistics more practical (otherwise one would have had to pick various time points and determine function of the hearts at all of those many time points). If a heart had died before 180 minutes, then its function was zero for the rest of the time points to 180 minutes and this would bring down the average of the group, which was the way of including all hearts and getting an idea of function of the *entire group*. One hundred and eighty minutes was chosen as this was close to the average survival time of the positive controls (of about 170 minutes).

In the "working heart literature", whether it is with rat, mouse, rabbit, or pig hearts and whether it is for studies of rejection, physiology or other, there are a number of different equations used to describe heart function (3-6). Some of these include cardiac work defined as cardiac output times peak blood pressure; rate pressure product defined

as heart rate times peak blood pressure; and stroke work index defined as (cardiac output times mean blood pressure) all divided by (heart rate times heart weight). In developing our model, it became quite apparent that "healthy" hearts had on average a heart rate of 150-200 beats per minute, a high peak blood pressure (80-110 mm Hg), and a large cardiac output (>350mL/min). It was felt that all three of these parameters were important in giving an idea of overall function of the heart and thus, all should be included in the equation for measure of function. If all were not included, then there may be incorrect conclusions drawn. For example, cardiac work does not include heart rate directly in the formula. Often, a positive control heart undergoing rejection would have a slow heart rate (e.g. 60bpm) and thus a longer diastolic filling time. Manual measurements of function were done every 15 minutes for 6-10 seconds in our system. During a measurement time, because of the fact that the positive control heart was quite full of blood (from the longer diastolic filling time secondary to slow heart rate), the cardiac output one would obtain may be relatively high (e.g. 350mL/min). Also, the pressure generated by the heart (partly because of the distention of the heart with the longer diastolic filling time), may be quite high (e.g. 90mmHg). In contrast, a healthy negative control heart may beat at the normal rate of around 180 beats per minute. Because of the faster heart rate, there is less filling of the heart in diastole, less stretch on the heart and thus a lower peak pressure. Such a heart may have a cardiac output of 400mL/min and a peak pressure of 80mm Hg. If one used the formula CO x SBP, then the sick heart would have a value of: 350ml/min x 90mmHg = 31500mL/min • mmHg and the healthy heart would have a value of: 400ml/min x 80mmHg = 32000mL/min • mmHg. These two would not be statistically different yet, from observation, one knows

that heart one was "sick" and heart two was "healthy". If heart rate were now incorporated into the formula, then heart one would have a value of 350ml/min x 90mmHg x 60bpm = 1890000mL/min • mmHg • bpm whereas heart two would have a value of 400ml/min x 80mmHg x 180bpm = 5760000mL/min • mmHg • bpm which is much more representative of what was observed. One may argue that one can make the heart rate constant by pacing the heart and thus not have heart rate as an issue. This is valid; however, for some of the positive control hearts, the hearts could not be paced to keep a constant rate (though the pacer was working and other hearts could be paced); similarly, the pacer could not pace above 150 beats per minute and thus, for hearts beating at 180 beats per minute or more, the pacer was not helpful as one could not slow the heart rate down with the pacer. The next measure of function in the literature, rate pressure product, was also not a good choice because cardiac output was not included in the equation. The parameter to fall the quickest in the hearts was cardiac output, followed by blood pressure and finally heart rate. Not including cardiac output in the equation would again give an inaccurate representation of function on paper and in statistics. Lastly, stroke work index does include all three parameters in the equation; however, heart rate is in the denominator. This fact would actually make "sick" hearts look better than "healthy" hearts. For example, in the hearts described earlier, heart one (sick heart) would have a stroke work index of (350ml/min x 90mmHg)/60bpm = 525. Heart two (healthy heart) would have a stroke work index of (400ml/min x 80mmHg)/ 180bpm = 178. Thus the "sick" heart would look better than the "healthy" heart. In addition to the above points, the purpose of establishing an equation is to describe and mathematically analyze data. As one was interested in differences between different

groups and not absolute numbers, it was felt that as long as the same equation was used for all hearts in all the various groups, and the relevant parameters were included, it should not matter what the exact form of the equation was. For the above reasons, (CO x SBP x HR)/1000 was chosen as the measure of function.

v) Definition of Heart Death

During heart perfusions, there were some hearts which would function for hours but would function very poorly (e.g. heart rate ~ 50bpm with systolic blood pressure of ~ 15mmHg and a cardiac output of 3ml/min). There were other hearts that would function well for a long time and then acutely deteriorate and stop. It was difficult to compare these different hearts as clearly the former heart is "functionally dead". To overcome these problems, the definition of death was related to function such that once cardiac function ((CO x SBP x HR)/1000) was less than 10, the heart was considered dead. The value 10 was chosen based on the graph depicted in **Figure 3.19** which shows that at 10, the hearts are "dead". Using this value of 10 allowed one to get more accurate survival numbers.

B) Development and Validation of the Model as a Model of Hyperacute Rejection

A model of hyperacute rejection was developed as demonstrated by highly significant differences in survival, function and weight changes. As well, light microscopy and immunofluorescence demonstrate findings consistent with hyperacute rejection in the positive control hearts. Creatine kinase (CK) changes were not significantly different in the groups though there was a trend towards higher CK rise in the positive control group. The reason for not obtaining significance probably relates to the fact that there was a lot of variability in the survivals of the positive control hearts

combined with the fact that the CK samples may have been taken from different places for different hearts. Usually the CK sample was taken from the pulmonary artery which would have the coronary artery blood return and thus the highest concentration of whatever came from the heart. Occasionally however, there was not enough blood return in the pulmonary artery to get enough sample; thus a sample was obtained from elsewhere in the circuit (which would likely have a more dilute concentration). These factors along with the fact that the positive control heart survivals were variable would give large variances and thus difficulty achieving statistical significance for CK changes. Measuring absolute numbers for CK changes (versus rates of change which is what was measured) was not accurate because as time went on, CK would rise and thus if absolute numbers were used, the negative control hearts (which all lasted a long time) would have very high CK levels and this would give the wrong message. For this reason, CK rate of change was used as opposed to absolute numbers. For the immunofluorescence, there was C3 binding in essentially all hearts including the negative controls. C3 is known to have an unstable thioester bond and it can become activated by many nonspecific factors which can lead to tissue deposition (7). Thus, C3 binding in the hearts is likely non-specific (not rejection related) and is probably not a useful factor to measure. C1q is a better measure since it is specific for antibody mediated complement activation (7) and would not be seen if complement was activated by the alternative pathway. In the pig-to-human combination, only the classical complement pathway is important (8-10) and thus C1q detection is a good test. If one was interested in complement depostion (regardless of pathway), then a better measure may be to stain for C5b-C9 (the membrane attack

complex) which is the final common pathway of complement activation for both the classical and altrernative pathways.

It is intuitive that technical factors may influence survival of working hearts. Data examination did not reveal any one predictor of survival in the factors looked at (**Table 3.1**); however, it was decided to continue to analyze technical factors. In general, one would expect that longer operative time, longer ischemia time, shorter time for the heart to stabilize (Langendorff time), more defibrillations, more air getting into the heart, lower pH, and lower hematocrit would all negatively effect survival. In addition, if potassium concentrations are too high (>5.0 mmol/L) or too low (<3.0 mmmol/L) this would negatively effect cardiac function. Lastly, if calcium concentrations were too high or low, then this may also effect cardiac function. **Table 3.2** demonstrates that the negative controls should have performed more poorly compared to the positive controls based on the above points. The fact that the opposite is true helps strengthen the fact that a good model of hyperacute rejection has been established.

There are some porcine working heart models to study hyperacute rejection present in the literature (5,6,11-19). Reviewing the literature and comparing it with our model shows that our model has some advantages compared to some of these other models. Some investigators either did not use a negative control or used allogenic pig blood (as opposed to autologous pig blood) for their negative controls. It is important to have negative controls to use as a standard. In our early studies, allogenic pig blood was used as well; however, it was found that survival and function were not optimum. With allogenic pig blood, survivals were at best ~240 minutes compared to ~420 minutes at best for autologous pig blood. This difference in survival time is important for this

project as some of the positive controls in this study lasted into the 200+ minute range. A few of thr allogenic pig blood perfused hearts demonstrated what was felt to be evidence of hyperacute rejection on gross pathologic exam (hemorrhage and thrombosis). The reason for the poorer survival and some of the gross pathologic changes in the allogenic pig blood group may be secondary to blood group incompatibilites between the allogenic blood and the pig heart. Using a negative control to determine ideal parameters for our system and using autologous pig blood are advantages of our model. Other advantages of our model is that technically it is easier and more practical. Some investigators had prolonged (~50-55 min) ischemia times versus our 6-8 minute time. Others needed volumes over one liter to run their perfusion system whereas ours only needed 350mL (minimum) and 400mL (optimum). This fact is very important since if one is using human blood and one needs to have a high concentration of human blood in the system, then the ability to get enough human blood to do enough replicates may become a problem. It was not clear all the time, but it appeared (at least for some of the literature), that one individual's blood was used for one heart. The potential problem with this is that there may be something about an individual that may bias results (e.g. other unusual antibodies). We did hearts using individual's blood for a heart but we also did very many hearts using relatively large pools of blood to try to minimize any biases that any one individual may have had. Lastly, in general there were more replicates per group in this study than others in the literature helping to increase the confidence in our findings. Thus, we felt our model has advantages compared to others.

Investigation of Various Factors Related to Hyperacute Rejection and Survival of Porcine Working Hearts

i) Human Red Blood Cells in Porcine-to-Human Xenotransplantation

The findings related to human red blood cells (hRBC) are quite interesting and novel. The exact reasons for the variability in the survivals of the hearts perfused with just washed hRBC (**Figure 3.11**) and the pathologic findings in some of the hearts of hemorrhage and thrombosis is not clear. It was shown via ELISAs and other tests that there was no antibody nor complement present in these RBC perfused hearts; and thus, this is not the explanation. It is known that processing (e.g. washing with saline, centrifuging, etc.) and storing (especially at 4°C) RBC can change their ability to agglutinate suggesting that their cell surface changes (20). Perhaps some of the red cells became "more sticky" with processing whereas others did not (due to subtle changes in processing) and thus, some red cells would stick to pig endothelial cells leading to endothelial cell activation with subsequent changes of thrombosis/ hemorrhage and heart failure. It is also known that hRBC are larger than pig RBC (21-23). Perhaps with the processing of the RBC, some cells became larger whereas others did not (due again to subtle changes in processing) and thus there were "large sticky" cells which lodged into the small blood vessels of the pig heart leading to problems. These factors will need to be tested in the future. Ways to test these factors include processing red blood cells (carefully and consistently), measuring the size of red blood cells (under a microscope) before and after processing and co-culturing the red cells (before and after processing) with pig endothelial cells and then measuring markers of attachement to endothelial cells

and/or endothelial cell activation. For some of the hearts that survived a very short time, it was thought that there may have been antibodies attached to the red blood cells that may have come off the red blood cells during perfusion of the hearts and bound to the pig hearts leading to rejection. This was speculated because it had been observed that adsorbing human plasma on processed human red blood cells did lead to depletion of various amounts of total antibody from the plasma. Thus, immunofluorescence examination of the hearts perfused with only red blood cells was done looking for human IgM and IgG deposition, but none was found. Thus, it is likely not antibody causing heart failure but structural red blood cell issues as discussed above.

Whole blood (plasma plus RBC) in general did improve function and survival as revealed by the differences in human plasma only versus human whole blood perfused hearts (**Figure 3.12-3.14**). We do know that the reason for this difference was not the anti-α-gal antibody level (**Table 3.5**). The reasons for the difference likely include: better oxygen delivery to the hearts facilitating better function (can undergo high energy producing oxidative metabolism better). This may have enabled the whole blood perfused hearts to withstand more injury before failing. Also, as mentioned earlier, it was found that human plasma adsorbed with human red blood cells did have a drop in total antibody pre to post adsorption. Thus, perhaps during perfusion of the hearts with whole blood, some of the antibodies bound to the processed human red blood cells and were therefore not available to attack the heart leading to longer survival. This finding may have clinical applicability. It is known that transfusion of red blood cells prior to allotransplantation in humans can help delay graft rejection (24,25). It may be beneficial for patients

undergoing xenotransplantation to receive processed red blood cell transfusion prior to the actual transplant procedure to help delay rejection.

Even though red blood cells in general improved survival (compared to plasma alone), there were still hearts that survived poorly. Different types of red blood cells (either O red blood cells, the universal blood donor) or type specific (A, B, AB) red blood cells had been used during the perfusions. It was hypothesized that the type of red blood cell was important and thus O versus non-O red blood cell groups were analyzed. The findings of the benefit of O red blood cells (**Figure 3.15**) was very interesting and clinically applicable. The differences are related to the red blood cell and not the anti-α-gal antibody levels (**Table 3.6**). O red blood cells are the universal red blood cell donor and so clinically, the transfusion of the RBC mentioned above should specifically be O red blood cells. O red blood cells do not express the A or B antigens present on people who are of other blood groups. It is known that as red cells age, they express the α-gal antigen and through this, are removed from the circulation (26-28). It is also known that diseased red blood cells express α-gal antigen and through this are involved in other disease (26,29,30). Perhaps with red blood cell processing, the red blood cells express α-gal antigen. Since O red blood cells do not have A or B antigen occupying sites on the membrane, perhaps more α-gal antigen can be expressed on O red blood cells, thus more α-gal antibody from plasma can be bound to the red blood cells leaving less available to cause rejection. This hypothesis could be tested by processing red cells of the different blood groups and measuring the amount of hemagglutination caused by an α-gal lectin (BSIB4) in serial dilutions. The hypothesis would be that O red blood cells would have more α-gal antigen expressed and thus would titrate further with the BSIB4 lectin

compared to the other blood group red blood cells. Alternatively, processed red blood cells of the different blood types could be used to adsorb human plasma and then the percent depletion of anti-α-gal antibody (if any) could be measured for the different red cell types to see if O red blood cells express more α-gal antigen. There is evidence in the literature (31,32) that O red blood cells are different from other red blood cells.

ii) Predictors of Rejection:

The final important points relating to our model deal with predictors of rejection. The factors examined were the ones that would be expected to be important for hyperacute rejection – antibody levels and complement (CH50). Initially, regression analysis using all positive controls (regardless of red blood cell type) was done. Subsequently, the data based on O or non-O red blood cell type was analyzed. It was important to do this as O red blood cells effect survival. According to thr regression analyses (**Tables 3.4(a-d)**), the only significant predictor of hyperacute rejection in our model is the anti-α-gal IgM and anti-α-gal IgG levels. The minimum amount of anti-α-gal IgM needed to get rejection was calculated. From the literature (33) it is known that anti-α-gal IgM comprises about 4-8% of total IgM. The total IgM in the plasma tested in our study was 1.0 g/L; thus (using 5% as the value for anti-α-gal IgM), the amount of anti-α-gal IgM in our sample was 0.05 g/L or 50 mg/L. Doing various dilutions of the plasma, it was found that at 15% plasma (7.5 mg/L), one was able to get earliest evidence of rejection (**Figure 3.18**). The finding of the importance of anti-α-gal IgM and anti-α-gal IgG levels supports evidence from the literature (2,34-40) which emphasizes the importance of anti-α-gal antibody as being the most important antibody for hyperacute rejection in the pig-to-human combination. It is interesting that the CH50 (complement)

level was not a significant predictor of rejection in our model though it is known that complement is very important in hyperacute rejection (hyperacute rejection may be prevented by inhibiting complement) (41-44). Perhaps in our model, antibody causing endothelial cell dysfunction (e.g. vasospasm) leading to heart failure occurred more predominantly than endothelial cell death. There is evidence in the literature linking endothelial cell dysfunction to cardiac dysfunction (14,45). In conclusion, we have developed a valid model of hyperacute rejection has been developed which can be used for further investigations. Novel findings include the role of human red blood cells, the determination of predictors of hyperacute rejection and determining the minimum amount of anti-α-gal IgM antibody needed to cause hyperacute rejection. Other important findings include that examining C3 deposition is not very helpful and that sample collection at a consistent location is important. There were also important technical issues.

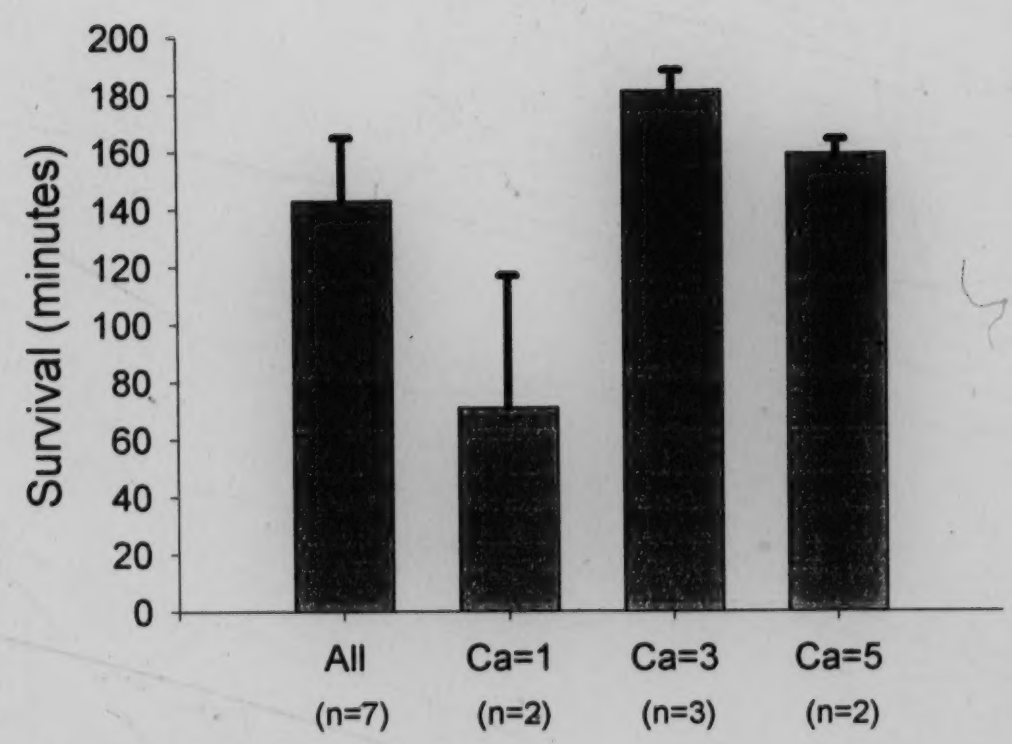


Figure 3.1 Survival of buffer (Kreb's Henseleit) perfused pig working hearts with different concentrations of calcium. Ca=1, Ca=3, and Ca=5 indicate $[Ca^{2+}]$ of 1.00, 3.00, and 5.00 mmol/L respectively.

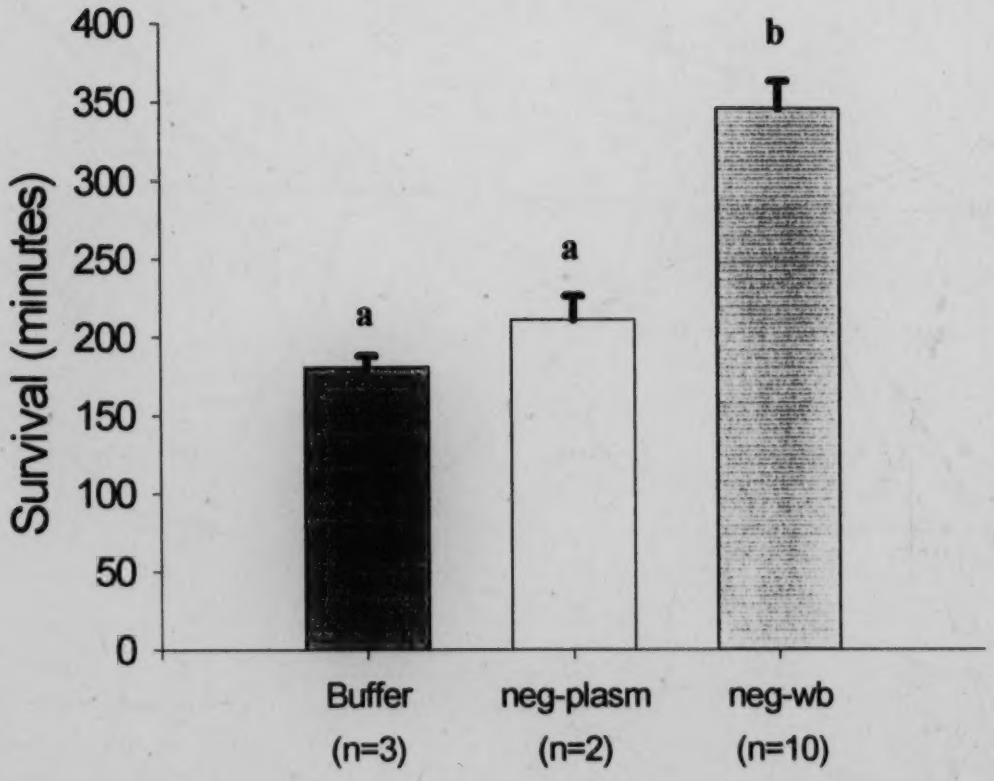


Figure 3.2 Survival of pig working hearts based on perfusate. Buffer is Kreb's Henseleit buffer with a calcium concentration of 3.00 mmol/L. Neg-plasm and neg-wb indicate pig plasma and pig whole blood respectively. Pig red blood cells significantly improve survival ($p<0.001$, ANOVA). Post-hoc Tukeys tests reveal that neg-wb is significantly different from buffer ($p<0.001$) and neg-wb is significantly different from neg-plasm ($p=0.021$). The symbols **a** and **b** denote statistically different groups. Data are expressed as mean ± SEM.

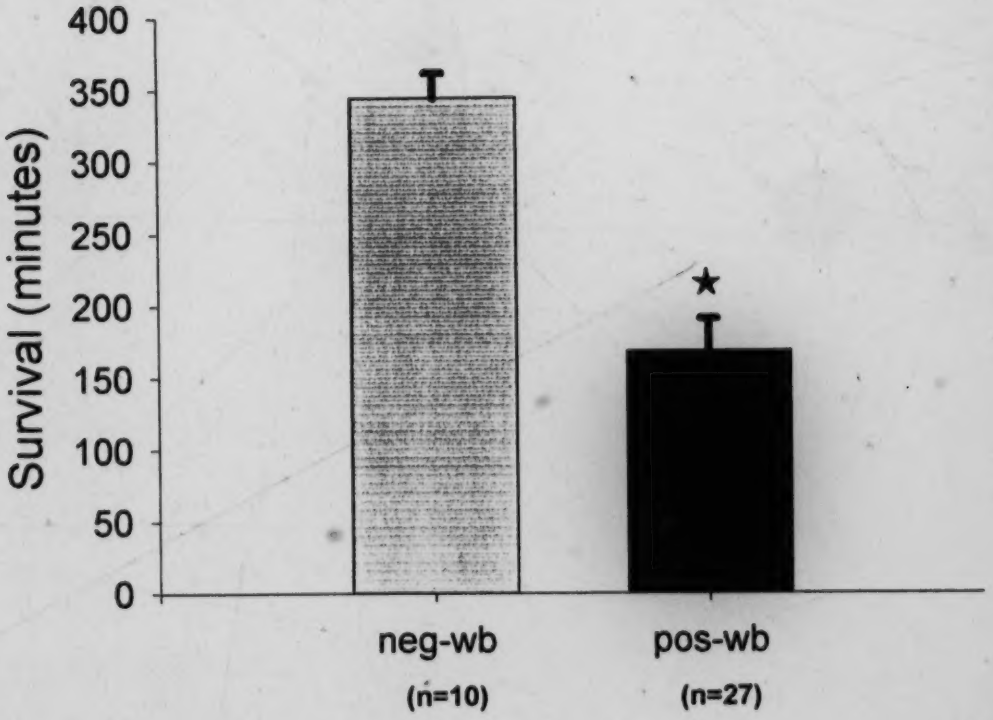


Figure 3.3 Survival of pig working hearts perfused with whole blood based on group. Neg-wb and pos-wb correspond to autologous pig blood (negative control) and human blood (positive control) respectively. ★ indicates significant difference ($p<0.001$, t-test). Negative controls survive significantly longer than positive controls. Data are expressed as mean ± SEM.

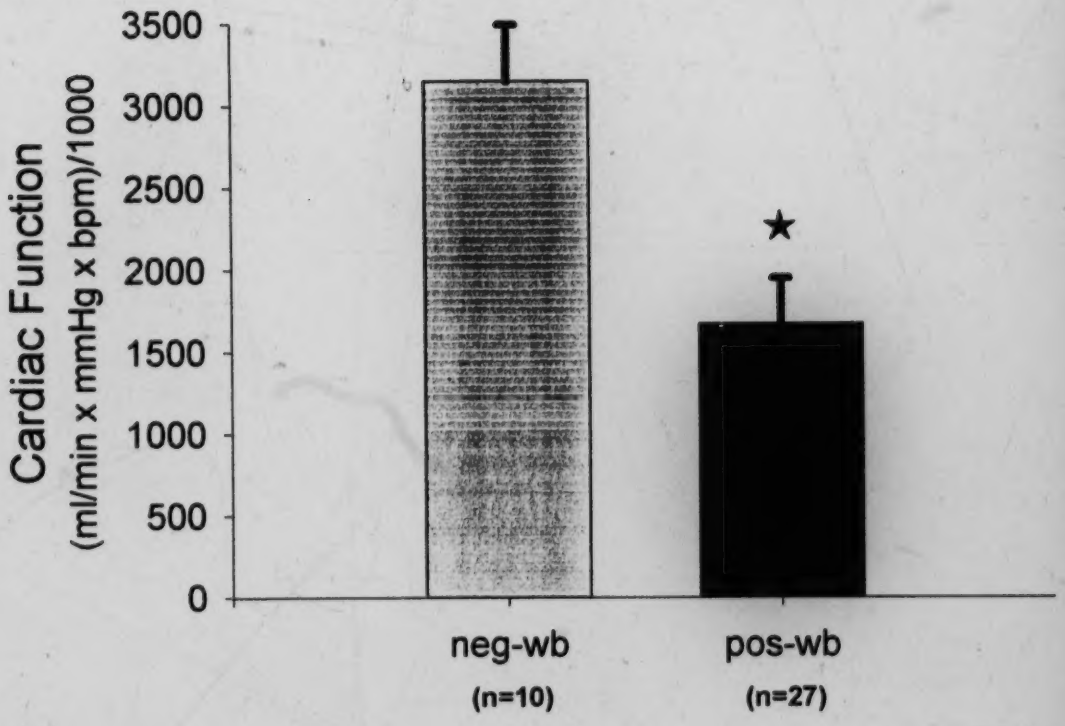


Figure 3.4 Average function of pig working hearts over the first 180 minutes of perfusion. Neg-wb and pos-wb indicate autologous pig blood (negative control) and human blood (positive control) respectively. ★ indicates significant difference ($p=0.006$, t-test). The negative controls function significantly better than the positive controls giving further support to this model as a model of hyperacute rejection. Data are expressed as mean ± SEM.

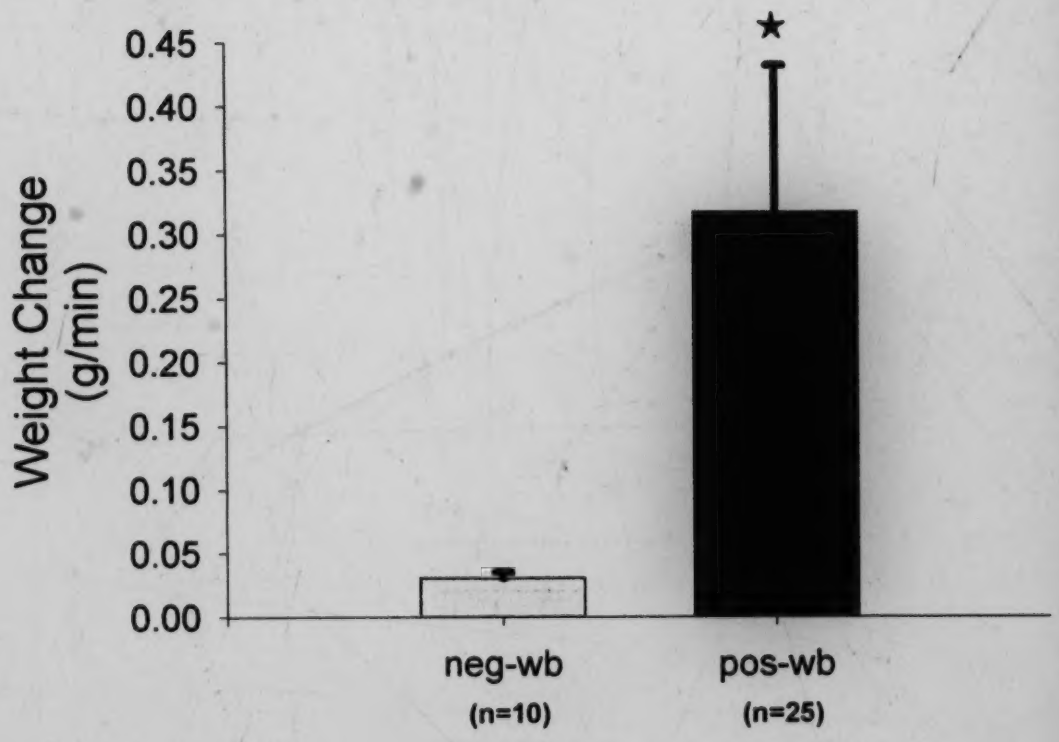


Figure 3.5 Weight change (as a measure of edema accumulation over time) of pig working hearts for various groups. Neg-wb and pos-wb correspond to autologous pig blood (negative control) and human blood (positive control) respectively. ★ indicates significant difference (p=0.001, Mann-Whitney U test). Positive control hearts acquire significantly more weight over time than negative controls, further supporting this model as a model of hyperacute rejection. Data are expressed as mean ± SEM.

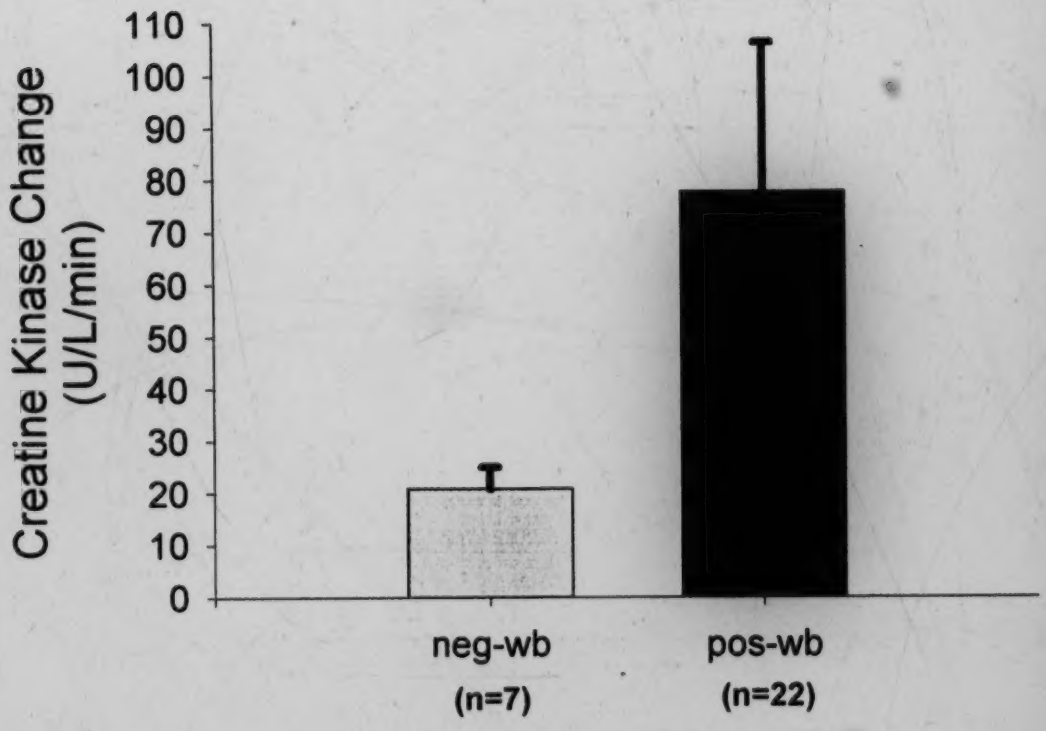


Figure 3.6 Creatine kinase (CK) change over time from pig working hearts for various groups. CK is a measurable protein released from damaged heart muscle. Neg-wb and pos-wb correspond to autologous pig blood (negative control) and human blood (positive control) respectively. The two groups are not significantly different from each other (p=0.110, Mann-Whitney U test). Data are expressed as mean ± SEM.

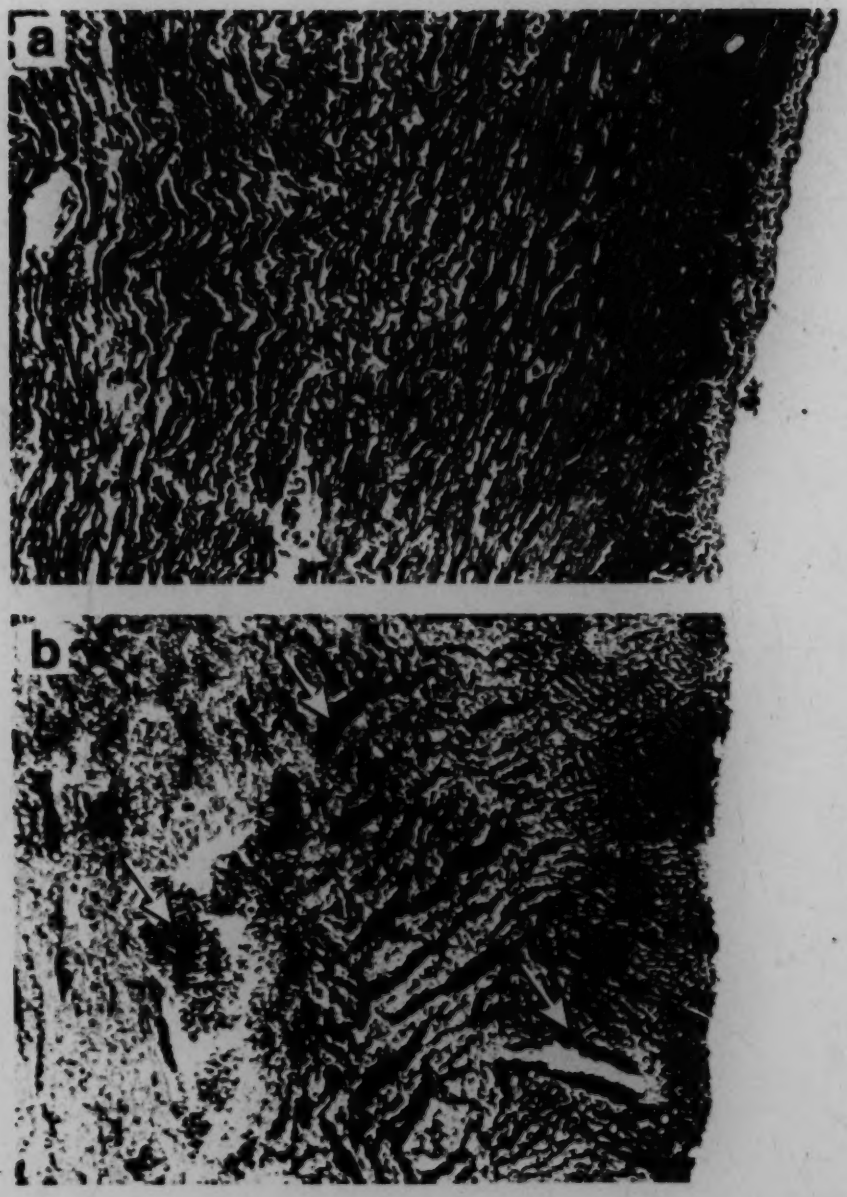


Figure 3.7. Hematoxylin and Eosin staining of pig working hearts perfused with a) autologous pig blood (negative control) or b) human blood (positive control). Positive controls show extensive hemorrhage (white arrows) and thrombosis (yellow arrow) consistent with hyperacute rejection. Magnification = 4x.

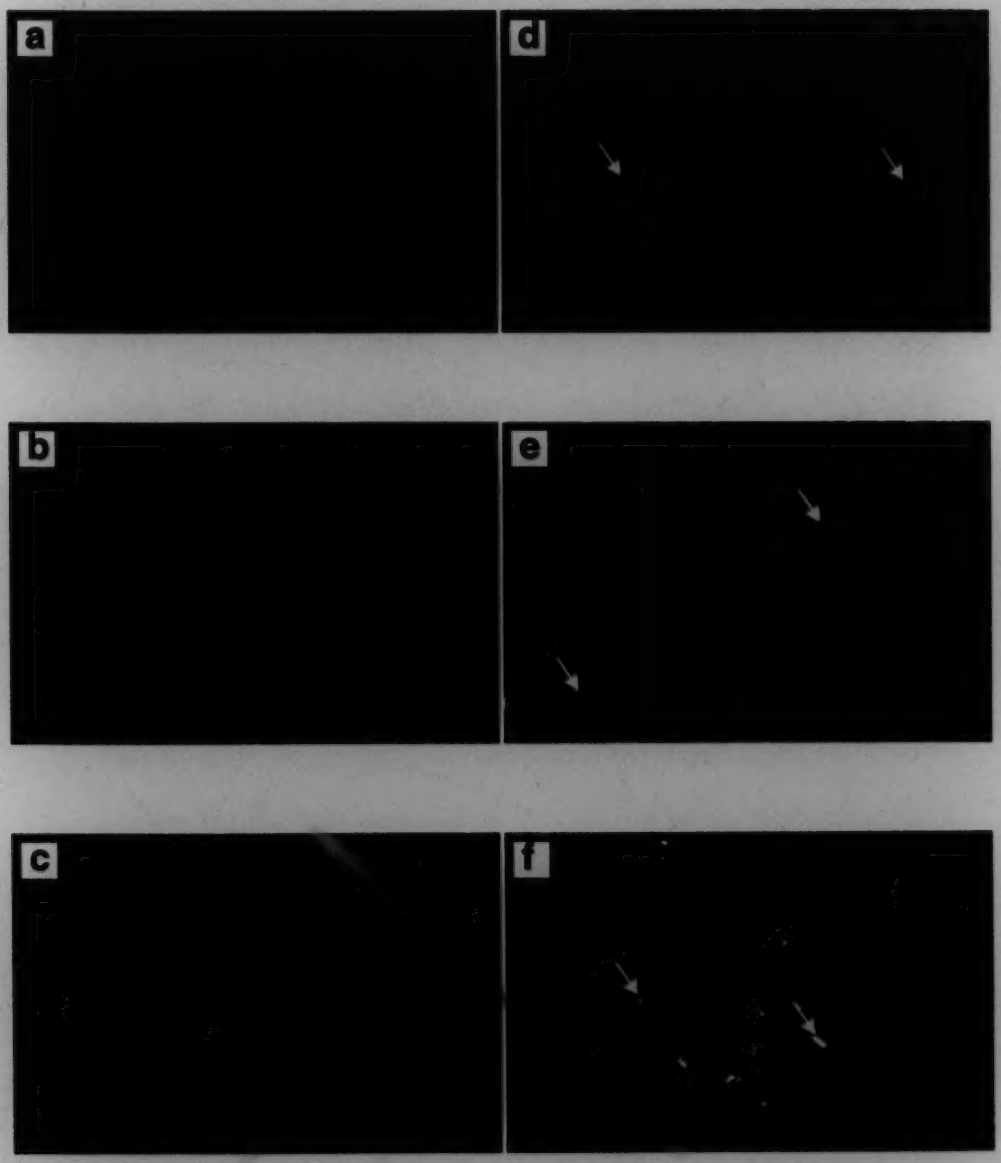


Figure 3.8. Immunofluorescence staining for human antibody and complement binding on pig working hearts. Negative control hearts: a) human IgM, b) human IgG, c) C1q complement protein. Positive control hearts: d) human IgM, e) human IgG, f) C1q complement protein. Positive control hearts show more binding of all three factors compared to negative control hearts (arrows). Magnification = 100x.

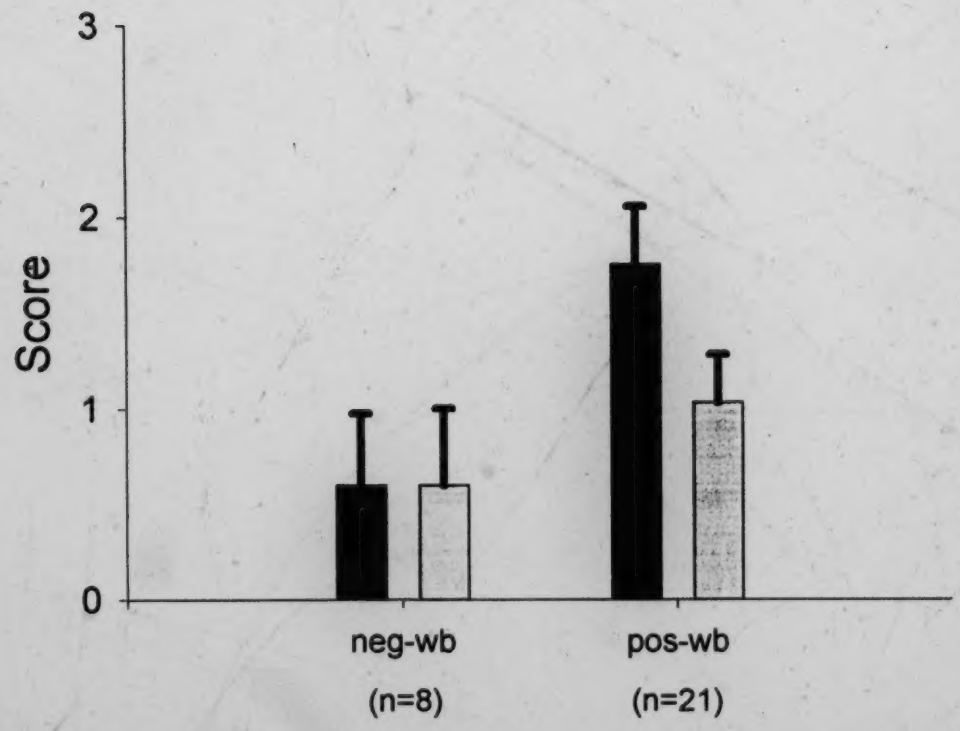


Figure 3.9 Light microscopy of pig working hearts stained with hematoxylin and eosin and scored for evidence of hyperacute rejection (hemorrhage ( ) and thrombosis (■)) via group. Neg-wb and pos-wb correspond to negative control whole blood and positive control whole blood respectively. There is more hemorrhage and thrombosis in the positive control group. Data are expressed as mean ± SEM.

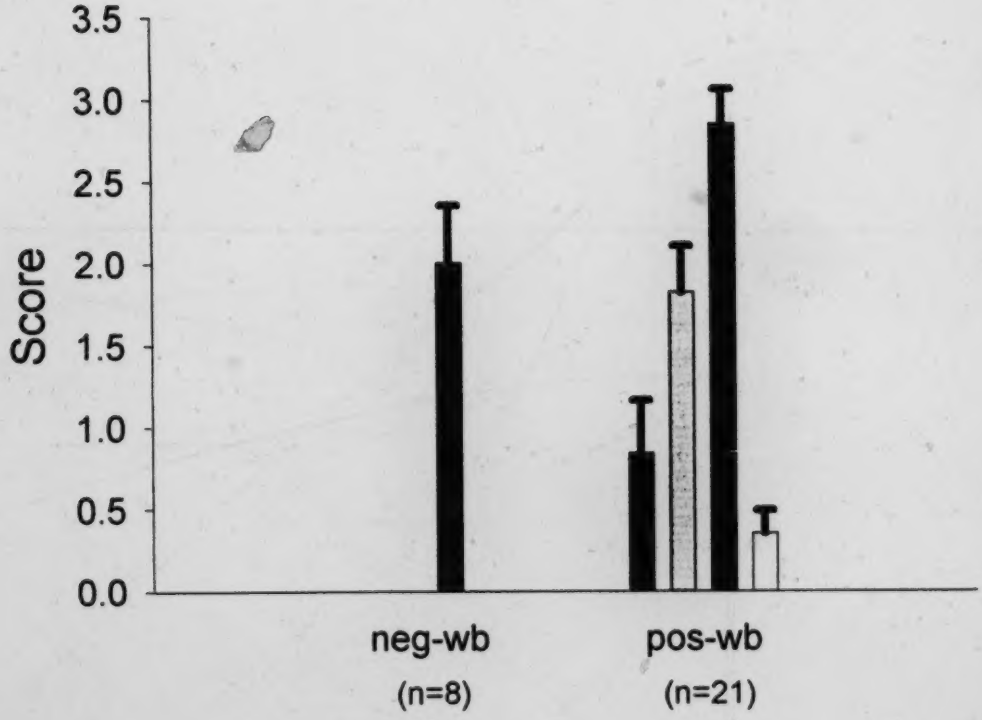


Figure 3.10 Immunofluorescence scoring for various factors for pig working hearts via group. Neg-wb and pos-wb indicate negative control whole blood and positive control whole blood respectively. ■=IgG, =IgM, ■=C3, =C1q. There is evidence of antibody and classical complement activation (C1q) on the positive control hearts. Data are expressed as mean ± SEM.

Table 3.1 Univariate linear regression analysis to determine which technical factors significantly correlate with survival and function of pig working hearts perfused with autologous pig blood (negative controls).

	SURVIVAL REGRESSION		FUNCTION REGRESSION		
Variable	Pearson Correlation Coefficient	P-value	Pearson Correlation Coefficient	P-value	n
Total OR time (min)	0.430	0.21	0.459	0.18	10
Total ischemia time (min)	0.179	0.62	-0.075	0.84	10
Total Langendorff Time (min)	0.343	0.33	0.189	0.60	10
No. of defibrillations	-0.191	0.60	-0.170	0.64	10
Air in heart (0=no, 1=yes)	0.230	0.52	-0.319	0.70	10
pH	-0.199	0.67	0.517	0.24	7
$[Ca^{2+}]$ (mmol/L)	-0.253	0.51	-0.139	0.72	9
Hematocrit (%)	-0.247	0.52	-0.612	0.08	9
PO_2 (mmHg)	-0.131	0.76	0.274	0.51	8
$[K^+]$ (mmol/L)	-0.029	0.95	-0.551	0.20	7

OR=operation, $[Ca^{2+}]$=calcum concentration, $[K^+]$=potassium concentration.
There are no significant linear corrlelations between the above variables and survival time or function.

Table 3.2 Comparison of technical factors between pig whole blood (negative control) and human whole blood (positive control) perfused pig working hearts.

Variable	Negative Control (n)	Positive Control (n)	P-Value (t-test)
Total OR time (min)	61.4 ± 5.5 (10)	29.6 ± 0.8 (27)	<0.001
Total ischemia time (min)	8.6 ± 1.0 (10)	6.4 ± 0.61 (27)	0.072
Total Langendorff Time (min)	26.9 ± 4.0 (10)	20.7 ± 0.9 (27)	0.162
No. of defibrillations	0.6 ± 0.2 (10)	0.21 ± 0.09 (27)	0.126
Air in heart (0=no, 1=yes)	0.2 ± 0.1 (10)	0.03 ± 0.03 (27)	0.257
pH	7.4 ± 0.01 (7)	7.44 ± 0.02 (27)	0.060
$[Ca^{2+}]$ (mmol/L)	3.2 ± 0.1 (9)	3.2 ± 0.07 (27)	0.941
Hematocrit (%)	13.2 ± 1.2 (9)	16.2 ± 0.5 (27)	0.012
PO_2 (mmHg)	429.1 ± 24.8 (8)	469.4 ± 13.2 (26)	0.151
$[K^+]$ (mmol/L)	6.0 ± 0.4 (7)	4.8 ± 0.2 (24)	0.003

OR=operation, $[Ca^{2+}]$=calcium concentration, $[K^+]$=potassium concentration.
One would have expected the negative control hearts to survive for a shorter time than positive controls due to the longer OR time, lower hematocrit, and higher potassium in the negative control group. Since this was not the case, the statistically significant variables are not biologically significant. Data are expressed as mean ± SEM.

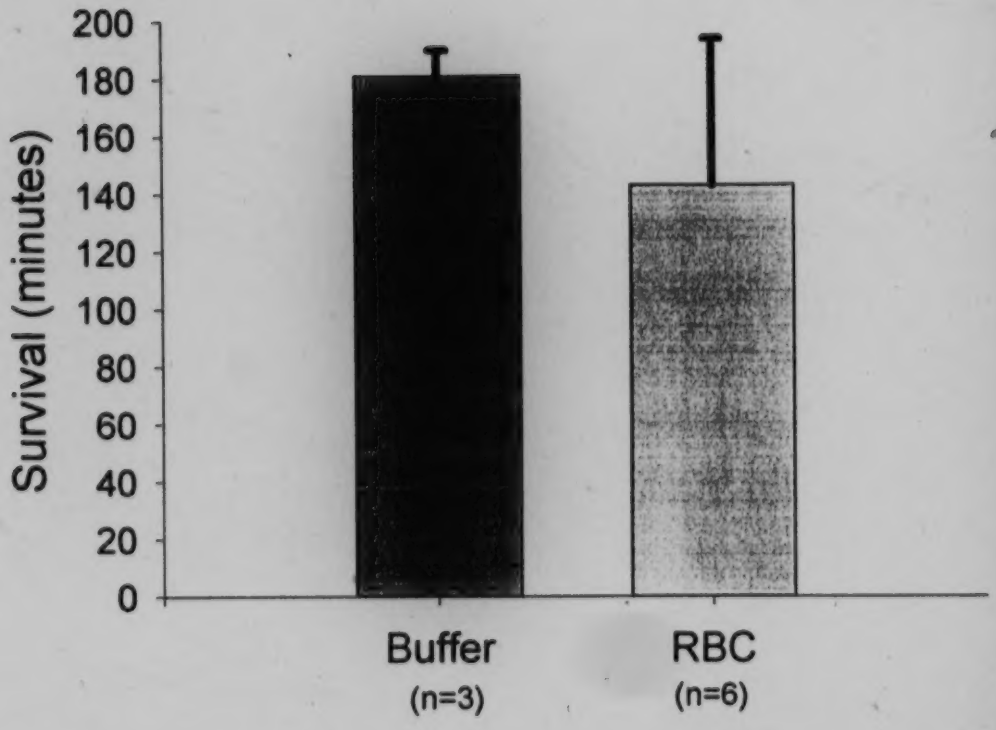


Figure 3.11 Survival of buffer (Buffer) perfused ($[Ca^{2+}]$=3.00 mmol/L) versus washed human red blood cell (RBC) perfused (no plasma) pig working hearts. The groups are not significantly different from one another (p=0.488, t-test). There is a lot of variability in using human red blood cells. Data are expressed as mean ± SEM.

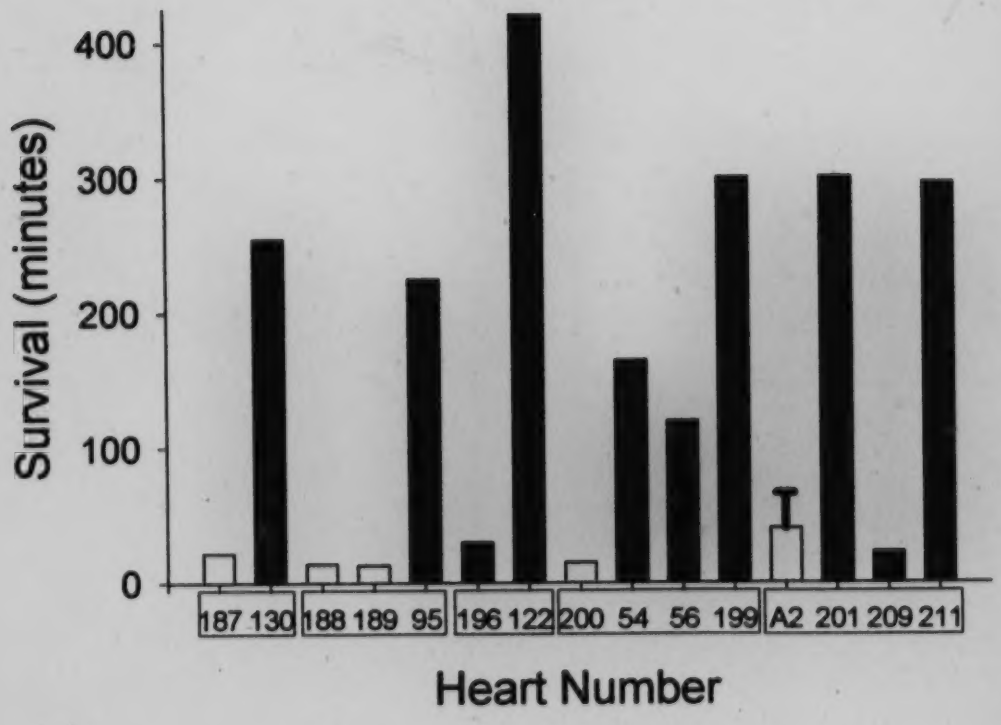


Figure 3.12 Survival of pig working hearts perfused with plasma ( ) or the same plasma and human red blood cells (■). Boxes define the hearts from the same groups. Although there is variability in survival with the use of red blood cells, hearts perfused with whole blood appear to survive longer. Data are expressed as mean ± SEM.

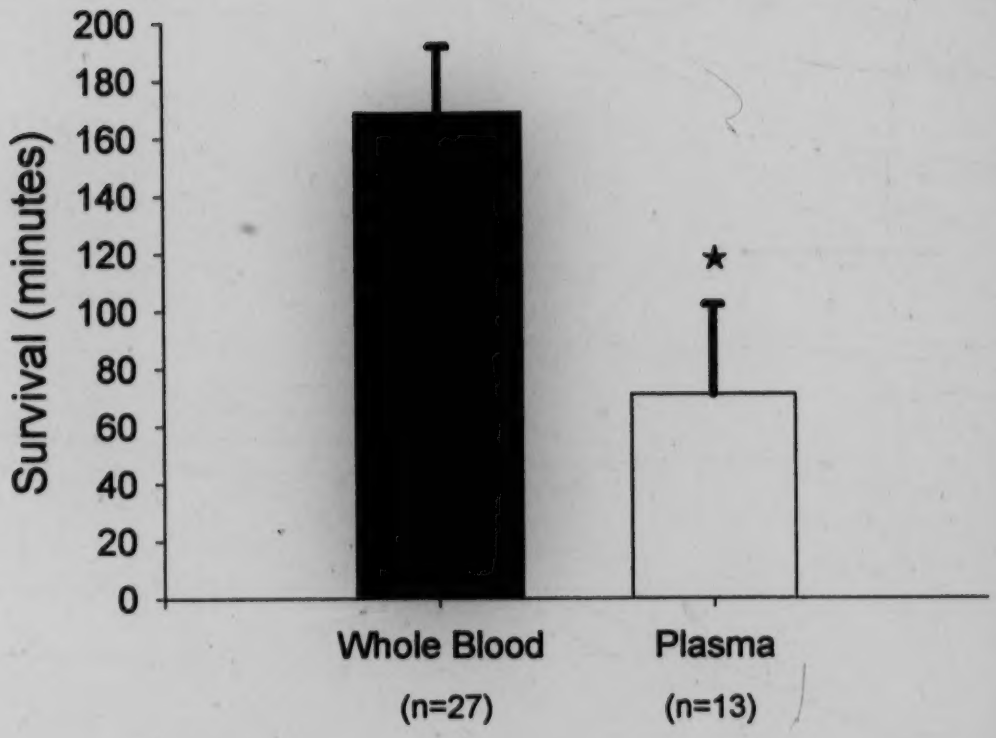


Figure 3.13 Survival of pig working hearts perfused with human whole blood (■) or human plasma only ( ). ★ indicates significant differences (p=0.017, t-test). Hearts perfused with whole blood survived significantly longer than hearts perfused with plasma only suggesting that in general, red blood cells improve survival. The data are expressed as mean ± SEM.

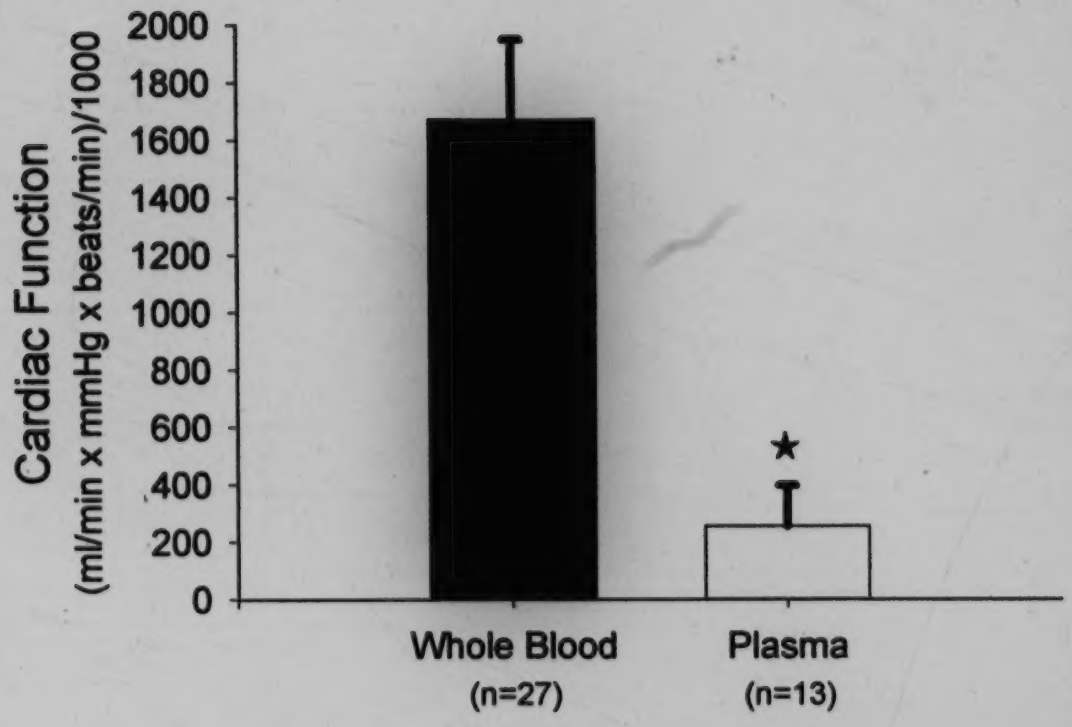


Figure 3.14 Average function of pig working hearts over the first 180 minutes of perfusion. Hearts were perfused with human whole blood (■) or human plasma only ( ). ★ indicates significant difference ($p<0.001$, t-test). The presence of red blood cells significantly improves cardiac function. Data are expressed as mean ± SEM.

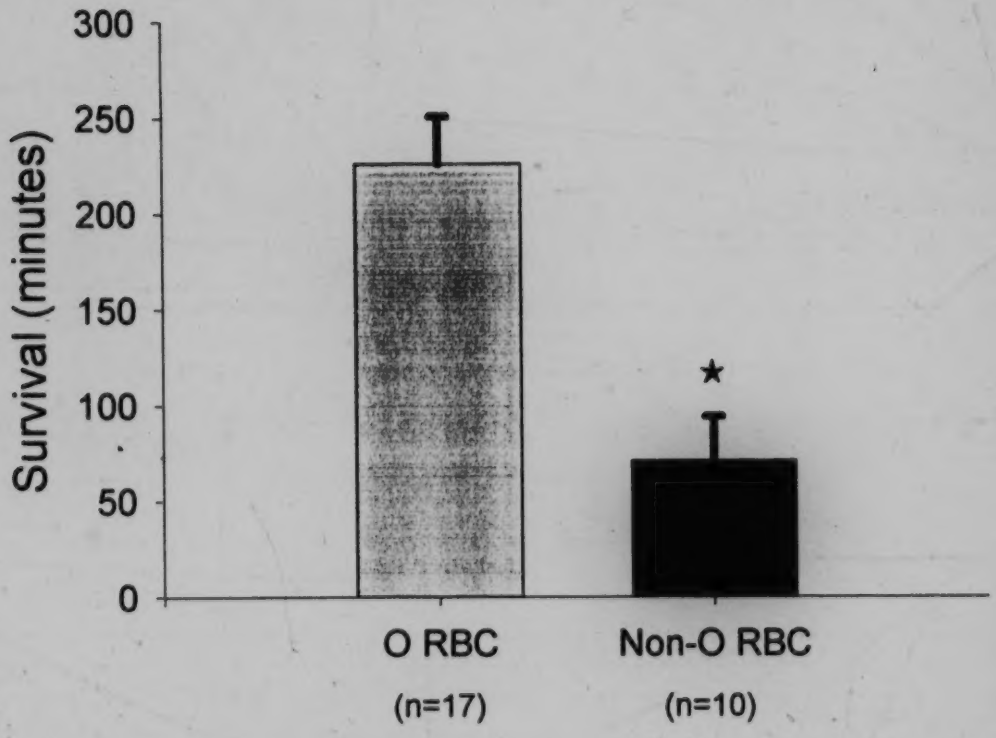


Figure 3.15 Survival of pig working hearts perfused with human whole blood having O red blood cells regardless of human plasma blood group (O RBC) or blood group A, B, or AB red blood cells as appropriate (Non-O RBC). ★ indicates significant difference ($p<0.001$, t-test). Using O-red blood cells significantly improves survival. Data are expressed as mean ± SEM.

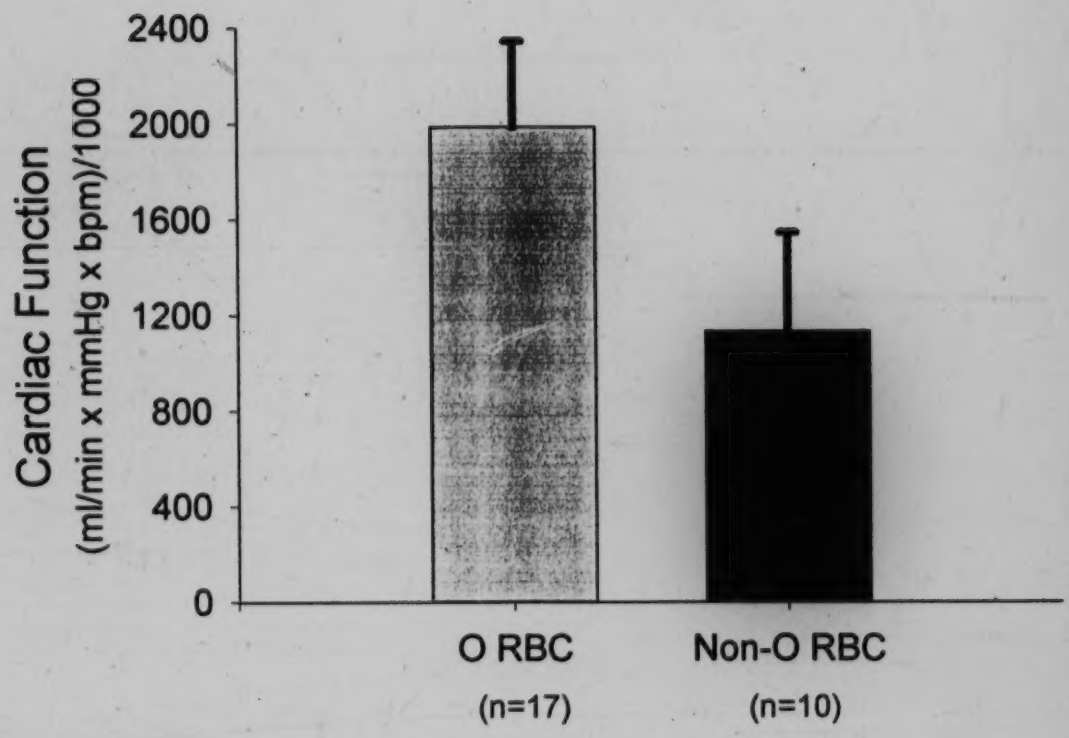


Figure 3.16 Average function of pig working hearts over the first 180 minutes perfused with human whole blood having O red blood cells (O RBC) or blood group A, B, or AB red blood cells (Non-O RBC). There is no significant difference between groups ($p=0.140$, t-test). In general using red blood cells helps function. Data are expressed as mean ± SEM.

Table 3.3 Comparison of technical factors between human whole blood perfused pig working hearts based on the type of human red blood cells used.

Variable	O-RBC (n)	Non-O RBC (n)	P-Value (t-test)
Total OR time (min)	27.9 ± 0.8 (16)	31.8 ± 1.2 (8)	0.014
Total ischemia time (min)	6.8 ± 1.1 (16)	6.1 ± 0.4 (8)	0.699
Total Langendorff Time (min)	18.9 ± 0.9 (16)	24.4 ± 1.8 (8)	0.005
No. of defibrillations	0.31 ± 0.15 (16)	0 ± 0 (8)	0.060
Air in heart (0=no, 1=yes)	0 ± 0 (16)	0.1 ± 0.1 (8)	0.351
pH	7.41 ± 0.03 (14)	7.47 ± 0.03 (8)	0.106
$[Ca^{2+}]$ (mmol/L)	3.39 ± 0.10 (14)	3.00 ± 0.07 (8)	0.011
Hematocrit (%)	16.8 ± 0.3 (14)	16.1 ± 0.9 (8)	0.408
PO_2 (mmHg)	468.4 ± 15.9 (14)	452.8 ± 31.2 (8)	0.624
$[K^+]$ (mmol/L)	4.87 ± 0.19 (14)	4.95 ± 0.54 (5)	0.854

OR=operation, $[Ca^{2+}]$=calcium concentration, $[K^+]$=potassium concentration.
Although there are statistical differences between some of the variables, these are not biologically significant.

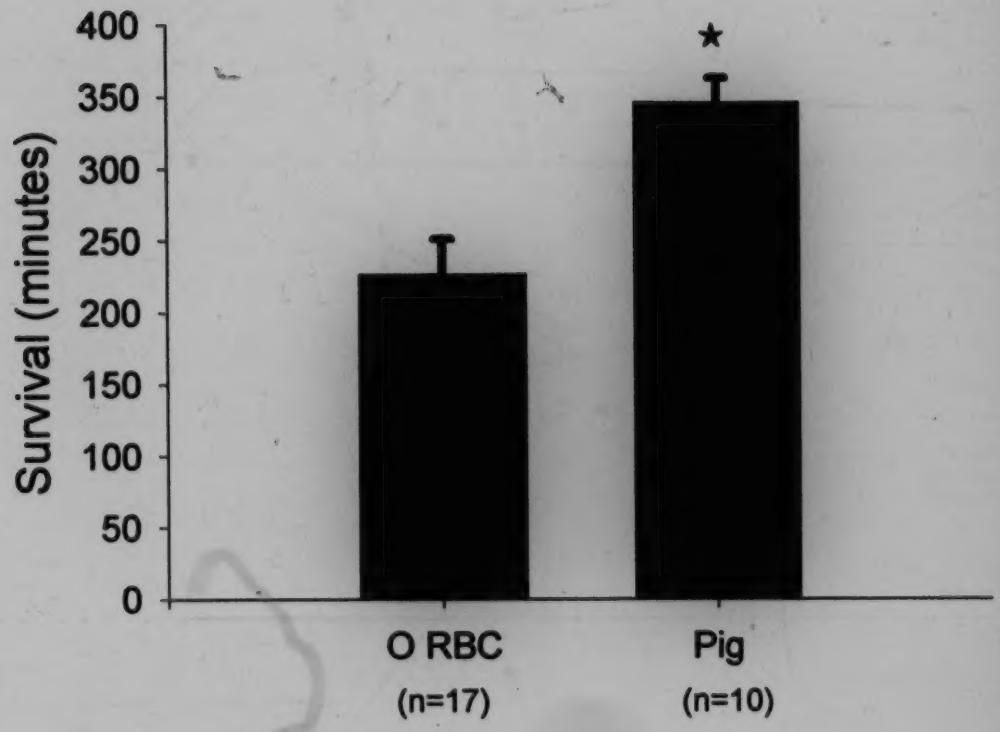


Figure 3.17 Survival of pig working hearts perfused with pig whole blood or human whole blood with O type red blood cells. Hearts perfused with O red blood cells survived almost as long as hearts perfused with pig blood. ★ indicates significant difference ($p=0.04$, t-test). Data are expressed as mean ± SEM.

Table 3.4(a) Univariate linear regression analysis of all whole blood positive control pig working hearts to determine which variables significantly correlate with survival and function.

SURVIVAL REGRESSION			FUNCTION REGRESSION			
Variable	Pearson Correlation Coefficient	P-value	Variable	Pearson Correlation Coefficient	P-value	n
Total IgM	-0.421	0.04	Total IgM	-0.442	0.03	24
Total IgG	-0.235	0.27	Total IgG	-0.223	0.29	24
Total IgA	0.096	0.67	Total IgA	0.137	0.54	22
CH50	0.280	0.23	CH50	0.271	0.25	20
α-gal IgM	-0.424	0.04	α-gal IgM	-0.481	0.02	24
α-gal IgG	-0.341	0.10	α-gal IgG	-0.328	0.12	24
anti-PAEC IgM	-0.254	0.23	anti-PAEC IgM	-0.422	0.04	24
anti-PAEC IgG	-0.019	0.93	anti-PAEC IgG	0.037	0.86	24
anti-A	0.111	0.61	anti-A	0.073	0.74	24
anti-B	-0.091	0.67	anti-B	-0.133	0.54	24

Total IgM, IgG, and IgA=human antibodies; CH50=measure of classical complement pathway activity; PAEC=pig aortic endothelial cells; anti-A and anti-B= blood group antibodies.
Blue values are significantly different.

Table 3.4(b) Multivariate linear regression analysis of all whole blood positive control pig working hearts to determine which variables significantly correlate with survival and function.

SURVIVAL REGRESSION			FUNCTION REGRESSION			
Variable	Pearson Correlation Coefficient	P-value	Variable	Pearson Correlation Coefficient	P-value	n
Total IgM	-0.412	0.43	Total IgM	-0.398	0.70	24
anti-α-gal IgM	-0.424	0.04	anti-α-gal IgM	-0.491	0.02	24
			anti-PAEC IgM	-0.346	0.44	24

The significant variables from table 3.4(a) were used in the multivariate regression.
Only the anti-α-gal IgM level is significant.

Table 3.4(c) Univariate linear regression analysis of whole blood perfused pig working hearts (positive control) having O red blood cells.

SURVIVAL REGRESSION			FUNCTION REGRESSION			
Variable	Pearson Correlation Coefficient	P-value	Variable	Pearson Correlation Coefficient	P-value	n
Total IgM	0.049	0.86	Total IgM	-0.339	0.20	16
Total IgG	0.035	0.90	Total IgG	-0.066	0.81	16
Total IgA	0.536	0.07	Total IgA	0.523	0.07	14
CH50	0.082	0.78	CH50	0.153	0.60	14
anti-α-gal IgM	-0.303	0.27	anti-α-gal IgM	-0.474	0.07	15
anti-α-gal IgG	-0.125	0.66	anti-α-gal IgG	-0.068	0.81	15
anti-PAEC IgM	-0.110	0.69	anti-PAEC IgM	-0.456	0.08	16
anti-PAEC IgG	0.113	0.68	anti-PAEC IgG	0.193	0.47	16
anti-A	-0.237	0.40	anti-A	-0.096	0.73	15
anti-B	-0.228	0.41	anti-B	-0.151	0.59	15

When O red blood cells are used, none of the above variables in significantly correlated with survival or function.

Table 3.4(d) Univariate linear regression analysis of whole blood perfused pig working hearts (positive control) having non-O red blood cells.

SURVIVAL REGRESSION			FUNCTION REGRESSION			
Variable	Pearson Correlation Coefficient	P-value	Variable	Pearson Correlation Coefficient	P-value	n
Total IgM	-0.065	0.88	Total IgM	0.001	1.00	8
Total IgG	-0.403	0.32	Total IgG	-0.311	0.45	8
Total IgA	-0.572	0.14	Total IgA	-0.498	0.21	8
CH50	-0.478	0.33	CH50	-0.542	0.27	6
α-gal IgM	-0.284	0.46	α-gal IgM	-0.264	0.49	9
α-gal IgG	-0.782	0.01	α-gal IgG	-0.743	0.02	9
anti-PAEC IgM	-0.032	0.94	anti-PAEC IgM	-0.050	0.91	8
anti-PAEC IgG	-0.290	0.49	anti-PAEC IgG	-0.332	0.42	8
anti-A	NS		anti-A	NS		
anti-B	-0.502	0.17	anti-B	-0.472	0.20	9

NS=no samples

When non-O red blood cells are used, only the anti-α-gal IgG level is significant.

Table 3.5 Anti-α-gal antibody levels for pig working hearts perfused with positive whole blood versus positive plasma only.

	Anti-α-Gal IgM	Anti-α-Gal IgG	n
Pos-Whole Blood	0.35 ± 0.05	0.81 ± 0.12	24
Pos- Plasma	0.30 ± 0.02	0.69 ± 0.05	13
t-test	*P = 0.290*	*P = 0.349*	

Table corresponds to figures 3.13 and 3.14.
Values have no units as they are standardized against a control serum
The groups have statistically similar levels of antibodies.
Data are expressed as mean ± SEM.

Table 3.6 Anti-α-gal antibody levels for pig working hearts perfused with positive whole blood based on red blood cell type.

	Anti-α-Gal IgM	Anti-α-Gal IgG	n
O RBC	0.23 ± 0.06	0.74 ± 0.14	15
Non-O RBC	0.45 ± 0.07	0.92 ± 0.22	9
t-test	*P = 0.125*	*P = 0.483*	

Table corresponds to figures 3.15 and 3.16.
Values have no units as they are standardized against a control serum.
The groups have statistically similar levels of antibodies.
Data are expressed as mean ± SEM.

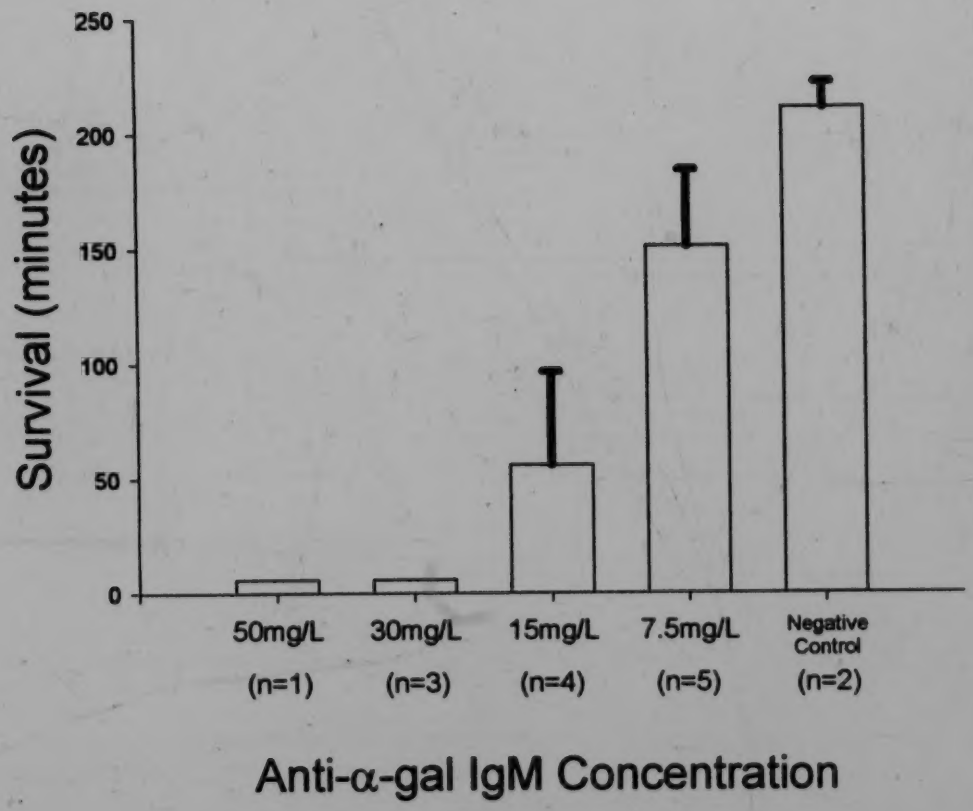


Figure 3.18 Determination of minimal concentration of anti-α-gal IgM needed to get rejection of pig working hearts. Pooled AB plasma used. Earliest evidence of rejection occurs at 7.5 mg/L of anti-α-gal IgM. Data expressed as mean ± SEM.

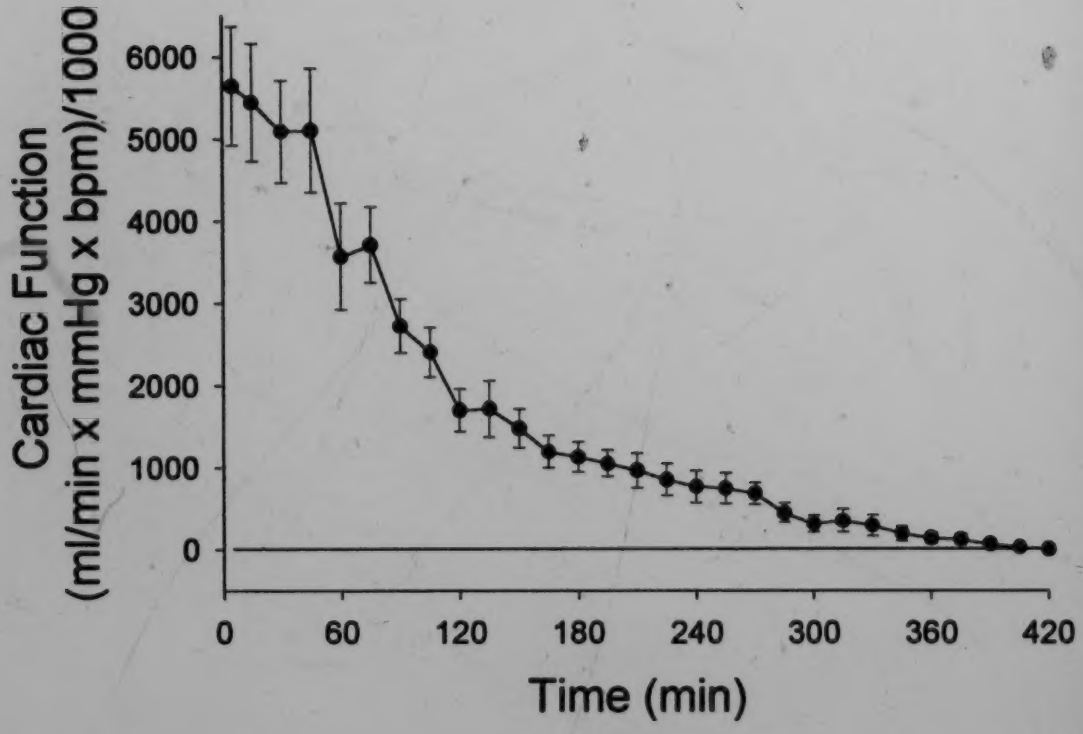


Figure 3.19 Average Cardiac function of pig working hearts (n=10) perfused with autologous pig whole blood (●—●). The blue line (—) indicates a function of 10 (ml/min x mmHg x bpm)/1000 (see text for explanation). Data are expressed as mean ± SEM.

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CHAPTER 4

Human ABO Blood Group in Porcine to Human Cardiac Xenotransplantation

Chapter 4: Human ABO Blood Group in Porcine to Human Cardiac Xenotransplantation

I. Introduction

The antibodies against the A and B antigens, which help distinguish blood groups in the ABO system, share a common origin and function with α-gal antibodies (1-3). Anti-A and anti-B antibodies pose a strong humoral barrier to allogenic transplantation (against the ABO barrier) in the same way that natural antibodies against Gal α1,3 Gal remain a significant problem to achieve successful xenotransplantation (1,4) The α-gal antigen and the B antigen are very similar in structure (**Figure 4.1(a)**), differing by only one fucose residue that is attached to the second α-gal sugar. While the A antigen is similar, it is less so. The potential exists for the anti-B antibody to cross react with α-gal antigens on porcine hearts, thereby augmenting the hyperacute rejection (HAR) response (**Figure 4.1(b)**). This could have profound effects on perspective xenograft recipients in the two most populated blood groups (A and O) in the Western hemisphere as both blood group A and O have naturally occurring anti-B antibody. Since anti-A is similar to anti-α-gal antibody (but less so), it may act as a weak agonist, it may not have any effect, or it may act as a competitive antagonist. This may also effect the success of xenotransplantation.

II. Objectives

1. To determine *in vitro* if the different human ABO blood groups have different levels of anti-α-gal antibodies.

2. To determine *in vitro* if the different human ABO blood groups have different levels of cytotoxicity against pig endothelial cells.
3. To determine in *ex-vivo* porcine working hearts if the different human ABO blood groups give different rates of rejection.

We hypothesized that there would be less anti-α-gal antibody, less cytotoxicity and slower working heart rejection in blood groups lacking the anti-B antibody (i.e: blood groups B and AB).

III. Methods (specific to this chapter)

From earlier studies (Chapter 3), it had been determined that human red blood cells have a role in xenograft survival. To study blood group issues properly, one needed to pool many people per blood group and test these pools (pool A, pool B, pool AB, pool O). Pooling would hopefully eliminate any individual biases that may alter heart survival and function and allow one to test the common factor(s) per blood group; which, for the purposes of this study, were the blood group antibodies - anti-A and anti-B. It was difficult to get all members of a particular pool to donate blood on the same day. For this reason, blood was collected when possible from the donor, and the plasma was frozen (quickly after collection). After the members of the pool had all donated, the "working pools of A, B, AB, O" were made and tested. Because of this process, it was not possible to have fresh human red blood cells of the correct blood type to use in the experiments. Human red blood cells were not used in these initial tests for the reasons given above, but in addition, our hypothesis involved the blood group antibodies which are present in plasma, and not using red blood cells was though to be beneficial because the differences

being looked for could be very subtle and any benefit offered from red blood cells may mask this difference. For the above reasons, it was decided to test the hypotheses for this section just using plasma. Once the hypothesis was prospectively tested using plasma, the whole blood positive control hearts (from Chapter 3) were reviewed via blood group for reasons outlined later.

When doing the experiments, it was observed that some hearts rejected extremely quickly once plasma was added, raising the question as to whether it was just the plasma or whether the hearts were dying from a technical problem. To eliminate this question, the pig hearts were initially perfused with buffer in working mode for 10 minutes before adding plasma while the heart was in working mode (see Chapter 2). Time 0 for data purposes was tha time the plasma was added.

All other methods are as outlined in Chapter 2.

IV. Results

A) Determining if there are *In Vitro* Differences in the Different Blood Groups:

Table 4.1 shows that in large pools of serum of each blood group (45 A, 18 B, 10 AB, 45 O), there was statistically less anti-α-gal IgM in blood group A and AB compared to blood group O. There is no statistically significant difference in the amount of anti-α-gal IgG present in any of the blood groups. **Table 4.2** shows that in regards to pig aortic endothelial cell (PAEC) cytotoxicity, blood groups AB and B are statistically similar whereas blood group AB is statistically different from blood group A and O. Note that blood group A is toxic even though the anti-α-gal IgM level is statistically the same as blood group AB.

B) Determining if there are *Ex-Vivo* Differences in the Different Blood Groups and the Possible Mechanisms:

Figures 4.2-4.6 demonstrate a highly significant difference between the blood groups in terms of survival, function, weight change, and creatine kinase change. In general, blood group B was the best followed by blood group AB. Blood group A and O were the worst.

The first issue that needed to be examined to ensure that differences seen were related to blood group differences was whether technical issues (**Table 4.3**) were similar between the groups. The only statistically significant difference between the blood groups that divided B and AB from A and O was the average potassium level. Blood groups B and AB had statistically higher potassium levels than blood groups A and O. However, the values were not biologically significant (the potassium values were within normal range) and also, the potassiums may just have been higher as hearts B and AB survived longer giving more time for myocyte potassium release. Thus, there were no technical reasons to explain the blood group differences.

The next possible explanation for the differences in the blood groups may be the presence of different levels of anti-α-gal antibody levels or other factors. **Table 4.4** shows that the important predictors, as determined in Chapter 3, the anti-α-gal IgM and anti-α-gal IgG levels for the blood groups are statistically the same. The "almost significant" level ($p=0.051$) for anti-α-gal IgG actually corresponds to a difference between blood group A and O which obviously does not explain the beneficial role seen by blood groups B and AB. There are a number of other statistically significant

differences between the blood groups; however, if the results of the multiple comparisons are examined, then most of the differences between the blood groups do not separate B and AB (well functioning hearts) from A and O (poor functioning hearts). Thus the statistically significant values probably have no biological significance but simply represent a mathematical phenomenon. The significant differences that do separate B and AB from A and O are the total IgM levels and the anti-B antibody levels. For total IgM, if one actually does a post-hoc Dunnett's test (as opposed to a post-hoc Tukey test), then blood group O versus B becomes non-significant. In addition, from the previous chapter, it is known that total IgM levels do not have a predictive role in determining survival, thus the total IgM difference between the groups is probably not important. The anti-B antibody however may be the explanation for the blood group differences.

As the anti-α-gal antibody levels were similar between the groups, we took the opportunity to see what the predictors of rejection were now. **Tables 4.5(a) and 4.5(b)** are the univariate and multivariate linear regression analyses on the data showing that the anti-B antibody is the overall significant variable – anti-B antibody has a deterimental effect on function and survival. Hemagglutination assays (n=18) of pooled porcine red blood cells using commercially bought (Sigma) purified mouse anti-B human antibody were done to help confirm the findings. Titrating to a dilution of 1/64, one found 4+ hemagglutination to that point (data not shown) – the positive controls (human AB red blood cells) were positive and the negative controls (human O red blood cells) were negative confirming the assay worked properly. All of the above suggest the anti-B antibody to be toxic.

To determine the role of anti-A was more difficult. The easiest method to address the role of anti-A would be to chose a simpler system (one in which one group had anti-A and the other did not). To dissect out subtle differences, more hostile, higher plasma concentration should be used. **Figure 4.7** shows that at 60% plasma concentration, there is a highly statistically significant difference for survival with blood group B being much better than AB. Blood group B (60% plasma) perfused hearts survived 218±45 min. versus 6±0 min. for blood group AB (60% plasma) hearts. Comparing the B-60% plasma concentration hearts to the B-15% plasma concentration (**Figure 4.7**) shows that the 60% hearts are statistically and biologically similar to the 15% hearts suggesting some protective agent in blood group B. Though the data is not shown, technical factors for the groups (B-60% and AB-60%) were similar and in terms of other factors (total antibodies, anti-α-gal IgM and IgG, etc.) the groups were similar except for the total IgG, total IgA, and anti-A antibody levels. Putting these variables in regression analysis only showed the anti-A antibody to be significant (data not shown). All of the above suggest some protective agent in blood group B plasma which is likely the anti-A antibody.

The positive control whole blood data (n=27) that was used in establishing and validating our model of HAR was retrospectively reviewed to see if differences in blood groups could explain some of the variability observed in the data. **Figures 4.8** and **4.9** show the survival and function of the hearts perfused by whole blood based on blood group. The important points of note are that firstly, there is no statistically significant differences between the blood groups for survival nor for function. Secondly, blood group O actually has the best survival and second best function (after blood group B) which is in sharp contrast from hearts perfused with plasma only. Thirdly, if blood group

O is ignored, then the general pattern seen is similar to that of the plasma only hearts (ie: blood group B is best, followed by AB and then A) suggesting that the pooled plasma heart data is representative. Knowing (from Chapter 3) that red blood cell type makes a difference, survival of the hearts based on blood group and red cell type (O RBC versus non-O RBC) was analyzed. **Figure 4.10** shows that if non-O red blood cells perfuse the hearts, then blood group A (has anti-B) does poorly and this is statistically significant. Unfortunately, with the small n number in B-nonO, one could not compare B-nonO to AB-nonO properly to determine if anti-A has a protective role. Perfusion with O red blood cells improves survival in all groups and statistically significantly so in blood group A.

Table 4.6 demonstrates that the anti-α-gal antibody levels are statistically similar in blood group A (O RBC versus non-O RBC groups) suggesting that O red blood cells are again protective and it is not different anti-α-gal antibody levels giving the differences in survival. There also were no biologically significant technical differences between the O RBC and non-O RBC blood group A perfused hearts (data not shown). **Table 4.7** demonstrates that the anti-α-gal antibody levels in the O whole blood group (**Figure 4.8**) is statistically the same as the anti-α-gal antibody levels in the O plasma only group (**Figure 4.2**). Thus, the superior performance of O whole blood is likely due to red blood cells.

Finally, the data were explored to determine whether there was a relationship between pig blood type, human blood type and heart survival. Pig red blood cells type to blood group O and A when using standard blood grouping methods of the Canadian Blood Services. It was found that out of 41 pigs that were typed, 88% were group O and

12% were group A. Unfortuneately, with this distribution, it was not possible to determine any relationship between pig blood type, human blood type and heart survival

V. Discussion

Because of the similarities between the α-gal antigen and the blood groups B and A antigens (**Figure 4.1(a)**), we hypothesized that there would be a human ABO blood group difference in porcine-to-human xenotransplantation.

A) There are *In Vitro* Blood Group Differences

Using pooled plasma for each blood group (to avoid introducing bias from one individual's plasma) it was found that blood group A and AB had less anti-α-gal IgM compared to blood group O (**Table 4.1**), and that blood groups B and AB had less cytotoxicity to pig endothelial cells compared to blood groups A and O (**Table 4.2**). It is interesting that even though blood group A had statistically similar anti-α-gal IgM as blood group AB and statistically less anti-α-gal IgM compared to blood group O; it actually had significant cytotoxicity (similar to O) suggesting that blood group A has a toxic factor, likely the anti-B antibody. Blood group B was statistically similar in regards to cytotoxicity to all groups (A, AB and O). The reason that it was not statistically better (though it was generally better) than blood group A is likely because blood group B has more anti-α-gal IgM than blood group A. The reason that blood group B was not statistically less cytotoxic than blood group O may be because this assay is not sensitive enough to detect subtle changes (e.g. such as a minor protective role of anti-A antibody). Though not statistically different, the level of cytotoxicity of blood group O is similar to

that of blood group A even though blood group O has significantly more anti-α-gal IgM antibody suggesting that there is a subtle inhibitory factor in blood group O (anti-A antibody?) giving some degree of protection.

Other *in vitro* studies have been done looking at blood groups. McMorrow *et al.* (5) found significantly decreased levels of anti-α-gal IgG reactivity in B and AB groups compared to A and O groups; though they only found a trend towards significance for anti-α-gal IgM. They also found no significant blood group differences for binding assays done using serum from different blood groups and pig peripheral blood mononuclear cells. McMorrow's group did say that their sample sizes were relatively small (n=12 per group) and there was a very large amount of variability between individuals. Neethling *et al.* (6) found a lot of variability in their *in vitro* assays but found that there was a trend towards lower anti-α-gal antibodies in blood group B sera; there were no significant differences in cytotoxicty to pig kidney cells in any of the blood groups although blood group B had lower cytotoxicity. Like McMorrow, they discussed the limitations of their assays of which sample size (n=5 per blood group) was one.

In our study, blood group O had the highest levels of anti-α-gal IgM compared to AB and A. Group B fell in between. There were no significant differences in anti-α-gal IgG between any of the groups. In addition, it was found that blood group A and O had more cytotoxicity than B and AB. The differences between all of these studies (ours, McMorrow's, and Neethling's) may be due to the fact that different conjugates were used as the ELISA target antigens as well as the fact that different cell lines were tested in the assays. Our assays had larger sample sizes for most of the blood groups (10 for AB, 18 for B, and 45 for A and O). Also, we used endothelial cells, which would be encountered

first by human antibodies perfusing a pig organ (as opposed to mononuclear cells or pig kidney cells). There have been other "binding type" assays done (1,3,7,8) looking at the anti-A, anti-B and anti-α-gal antibodies. Some have found that anti-A and anti-B do bind pig tissues/cells (1,7) whereas others have not found this (4,8) or have found conflicting results in their experiments (8). Galili (1) and others (6) have found that anti-α-gal antibody in blood group A and O individuals will bind B antigen. All of these studies suggest that there may be a blood group difference, and in fact, there is literature (2) discussing how the anti-α-gal, anti-A and anti-B antibodies are members of a distinct family of natural antibodies. However, presumably due to the fact that the *in vitro* assays done have had much variability in their findings, the xenotransplantation literature says that anti-ABO antibodies seem to play no role in rejection of pig organs (4,9,10). It is possible however, that the assay systems being used are not the correct systems to look for more subtle differences that may be present with blood groups. A solid organ system, looking at organ function may be a better system to view subtle differences. To date, no one (except for this study) has examined blood group differences in a physiologic, clincally applicable solid organ system.

B) There are *Ex-Vivo* Porcine Working Heart Blood Group Differences

Using different pools of plasma (that had similar levels of anti-α-gal antibody) it was found that pig hearts perfused with blood group B plasma gave the longest survival with the best function and that pig hearts perfused with blood group AB gave good survival but with much poorer function (**Figure 4.3**). Hearts perfused with blood group B and AB plasma survived 10-35 times longer than hearts perfused with blood group A and O plasma (**Figure 4.2**). The B and AB hearts also had less edema accumulation (weight

gain) and less creatine kinase rise compared to the A and O hearts. Because pooled plasma samples were used where the known common factor was the blood group; because the technical factors were biologically similar between the groups; and because the levels of anti-α-Gal antibody were similar for the groups, we conclude that there is a human ABO blood group difference for pig-to-human cardiac xenotransplantation. Using data from the plasma perfused hearts, it appears as though people who are blood group B are best suited to receive xenotransplants, followed by people who are blood group AB, A, and then O.

The possible mechanism that may explain the split between blood groups B and AB versus blood groups A and O is the anti-B antibody. As discussed in Chapter 1, blood groups A and O (but not B and AB) have the anti-B antibody and the B antigen is very similar in structure to the α-gal antigen structure. Thus, perhaps the anti-B antibody is attacking the porcine xenograft. Evidence to support this comes from the linear regression analyses done **(tables 4.5(a) and 4.5(b))** showing that only the anti-B antibody is significant in predicting survival in our plasma-only perfused hearts. Other evidence that the anti-B antibody is involved in rejection comes from our hemagglutination experiment showing that commercially bought anti-B antibody binds pig red blood cells. Lastly, the literature discussed the ability of anti-B antibody to bind α-gal antigen (1).

As function of the AB hearts was much poorer than the B hearts, we hypothesized that there may be a more subtle difference between these two blood groups that may be determined if more severe conditions were used in our "assay system" – porcine working hearts. We hypothesized that the anti-A antibody in blood group B may have a protective role. Using a higher (60%) concentration of plasma, it was found that blood group B

plasma perfused porcine hearts still survived statistically as long as the lower (15%) blood group B plasma perfused hearts (**Figure 4.7**). However, for blood group AB plasma, the xenograft survival time was 25 times lower using 60% plasma than when using 15% plasma. This suggested that there was something in blood group B plasma that was protective (the anti-A antibody?). Regression analysis revealed that the anti-A antibody is protective.

The reason that the anti-A antibody may be protective can be speculated on by looking at the antigen structures for blood group A and the α-gal antigen. (**Figure 4.1(a)**). It is noted that while there are two sugar residues that are different between the α-gal antigen and the A-antigen, the rest are the same. One of the different sugars is GlcNAc, and it is in a critical terminal position. It is possible that the anti-A antibody may act as a competitive inhibitor to the binding of anti-α-Gal antibody in blood group B plasma, and this protection is not available in blood group AB plasma. Evidence from the literature to support the hypothesis that anti-A antibody may have a competitive inhibitor role is that others have found that anti-A antibody can weakly bind to pig tissue, (4,11).

One may ask at this point that if anti-A antibody is protective, then why do blood group O plasma (has anti-A, anti-B, and anti-α-gal antibody) perfused hearts not survive very long. The possible reasons for this include the fact that the anti-A antibody may be out competed by the presence of both anti-B and anti-α-gal antibody (both these antibodies likely having higher affinity for the α-gal antigen). Also, it is known that the proportion of the higher affinity IgG (as opposed to the lower affinity IgM) anti-A and anti-B antibody present in O plasma is higher than in the other blood groups (12). These higher affinity IgG antibodies in blood group O may bind the α-gal antigen stronger

(compared to the IgM in other blood groups) leading to endothelial cell activation with its consequences of endothelin-1 release and coronary spasm with organ failure (13-16).

The whole blood data collected in Chapter 3 was retrospectively analyzed to see if there was a blood group difference to add support to our plasma only perfused heart findings. When all hearts were examined (**Figures 4.8 and 4.9**), there actually was no statistically significant differences between the blood groups even though the general pattern of B being the best followed by AB and than A was observed (if blood group O was ignored). The reason that there was no statistically significant difference may have been due to the presence of red blood cells helping the hearts and thus making the system less sensitive to subtle changes. When the hearts were examined based on type of red blood cell (**Figure 4.10**), significant differences in the non-O red blood cell groups were evident. As people would generally have their own blood type red blood cellscirculating in their bodies (and not O RBC), there is a blood group difference using whole blood with blood group A being the worst.

Hearts perfused with blood group O whole blood performed much better than blood group O-plasma only. Perhaps the presence of O red blood cells and the presence of the anti-A antibody help offer exceptional protection to the pig hearts.

A potential source of error is that the anti-α-Gal ELISA used to show that the anti-α-gal antibody levels were the same in the pooled plasma samples, may actually be detecting the anti-B antibody (via cross reaction). Though possible, it is probably not likely for a few reasons. Firstly, the α-gal antigen on the ELISA plate is a little more "stringent" as it has a Glc (not GlcNAc) as the third sugar and also, there is no fucose sugar present, thus, there is a two sugar difference that the anti-B antibody would have to

cross-react with for it to bind. Secondly, the ELISA conditions are set up to try to avoid cross reactive antibodies from binding (detergent in wash solutions, frequent washes, etc.). The last point to show that the anti-α-gal ELISA is specific is the fact that it was specifically tested for this. Human O plasma was adsorbed on human AB red blood cells and this lead to 100% depletion of the anti-B antibody but no depletion of the anti-α-gal antibody via our ELISA technique (n=16, data not shown) - thus our ELISA is specific.

The clinical implications of finding blood group differences for porcine-to-human cardiac xenotransplantation are great. Firstly, the information can be used to tailor immunosuppression/immunodepletion protocols for patients based on their blood group. This would allow less immunosuppression (with less immunosuppressive complications) for those likely to accept the xenograft more easily; but still allow adequate xenograft protection. Secondly, perhaps all patients (regardless of blood group) should be transfused with O red blood cells prior to transplantation; and in the future as well if the patient needs blood transfusion for whatever reason. The possible reasons for O red cell benefits was given in the previous chapter. Thirdly, it is known that ethnic groups have different blood group distributions in their populations (12,17). In particular, some Asian populations tend to have more blood group B individuals (~1/3 of population) compared to the British population (~1/8 of population) (12). Some Asian countries (like Japan) generally have cultural reluctance to use brain dead human donors (18). These countries may therefore benefit substantially from porcine organ use and they may end up being the most likely to not have major problems with rejection due to their blood group distribution.

The findings of this study are novel and important for the following reasons. Even though others have looked at blood group issues in xenotransplantation, their assays have all been *in vitro* assays (binding and cytotoxicity assays) which may have little relevance to whole organ issues and clinical xenotransplantation. Also, some of the investigators did not get significant results (maybe because their *in vitro* system was not as sensitive as an *ex-vivo* working heart system); did not have as large sample sizes that we have; and did not explore mechanistic issues (agonist role of anti-B and antagonist role of anti-A antibody). Our study examined whole organ function finding very significant results with clinical implications. We also briefly looked into mechanistic issues to explain why there are the blood group differences that we found as well as looking at differences related to perfusion with O and non-O red blood cells.

VI. Figures and Tables

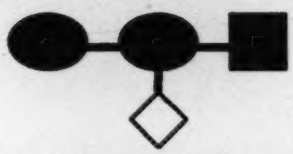
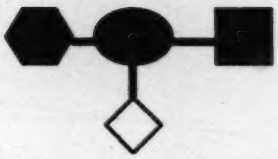
Antigen Structure	Antigen Name	Attacking Antibody	Blood Group of Attacking Antibody
	α-Gal	Anti-α-Gal	All blood groups
	B	Anti-B	Group A and O
	A	Anti-A	Group B and O

Figure 4.1(a) Diagrammatic representation of the α-gal, B, and A antigens. ●=galactose, ■=N-acetylglucosamine, ◇=fucose, ⬢=N-acetylgalactosamine.
Note the extensive similarity between the α-gal antigen and the B antigen. There is some similarity between the α-gal antigen and the A antigen.

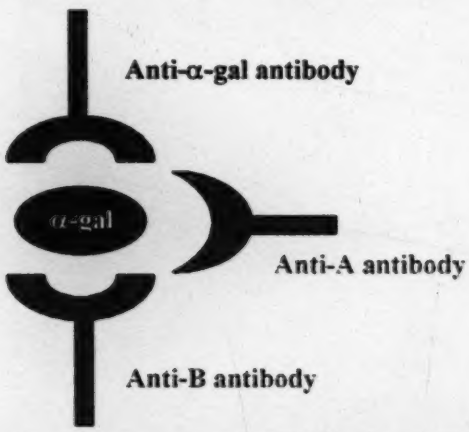


Figure 4.1(b) Diagrammatic representation of possible cross reactivities of the α-gal, B, and A antibodies. Since the α-gal antigen and B antigens are similar, the anti-B antibody may bind α-gal antigen on pig tissues and augment rejection. The A antibody has the potential to be a weak agonist or competitive antagonist.

Table 4.1 Anti-α-gal antibody levels in the different blood groups

Blood Group	Number in Pool	Anti-α-gal IgM	Anti-α-gal IgG	n
A	45	0.541 ± 0.03	0.769 ± 0.07	11
B	18	0.598 ± 0.06	0.794 ± 0.02	11
AB	10	0.455 ± 0.02	0.829 ± 0.02	11
O	45	0.717 ± 0.06	0.855 ± 0.03	11
	ANOVA	*P=0.001*	*P=0.430*	

No units are shown as results are standardized against a control serum to allow comparisons between ELISA plates. There is a statistically significant difference in the anti-α-gal IgM level between the blood groups. Post-hoc testing (Tukey) in this group showed O vs A p=0.032, and O vs. AB p=0.001 with all other combinations not being significant.

Table 4.2 Cytotoxicity against pig aortic endothelial cells in the different blood groups

Blood Group	Number in Pool	Pig Aortic Endothelial Cell Survival	n
A	45	0.88 ± 0.12	12
B	18	1.13 ± 0.07	4
AB	10	1.47 ± 0.29	4
O	45	0.89 ± 0.06	12
	ANOVA	*P=0.026*	

No units are shown as results are standardized against a control serum to allow comparisons between different plates. There is a statistically significant difference in pig aortic endothelial cell survival between blood groups. Post-hoc testing (Tukey) showed AB vs. A p=0.028; and AB vs. O p=0.038. All other comparisons are not statistically significant.

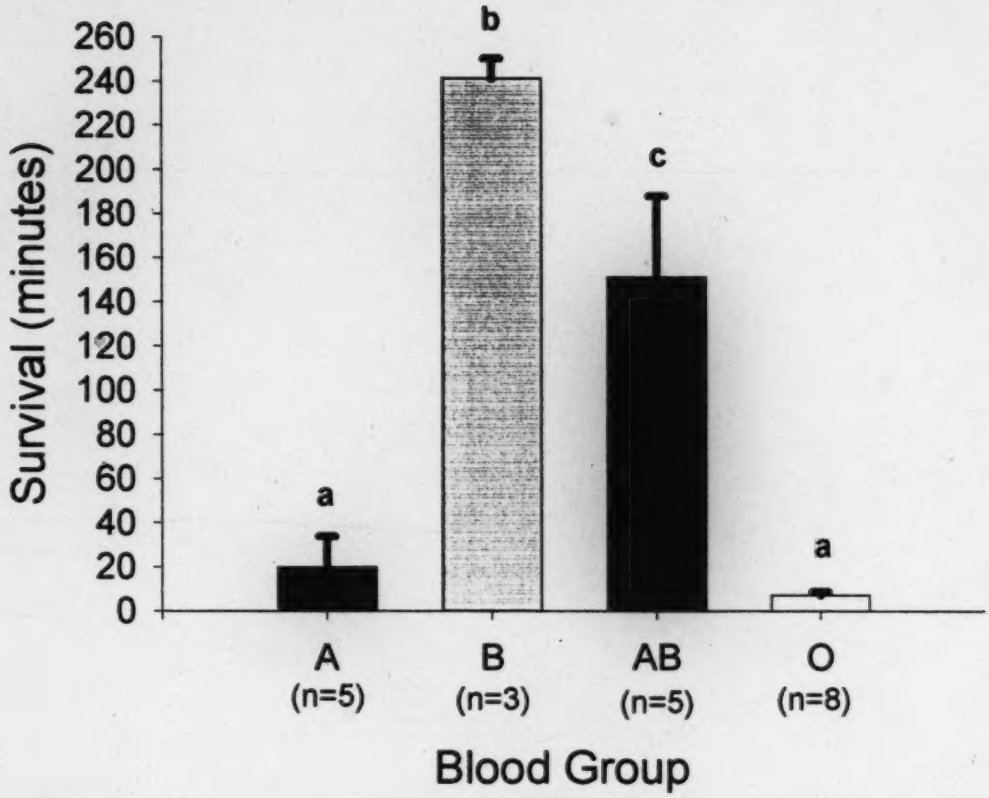


Figure 4.2 Survival of 15% human plasma perfused pig working hearts based on blood group. Each blood group has a minimum of 5 donors to form the blood group pool. The results are highly significant ($p<0.001$, ANOVA). Post-hoc Tukey tests show blood group B to be statistically better than A and O and blood groups A and O to be statistically similar. The p-values are: B vs. A, $p<0.001$; B vs. AB, $p=0.048$; B vs. O, $p<0.001$; AB vs. A, $p=0.001$; AB vs. O, $p<0.001$; O vs. A, p=ns. The symbols **a, b,** and **c** indicate statistically different groups. Data are expressed as mean ± SEM.

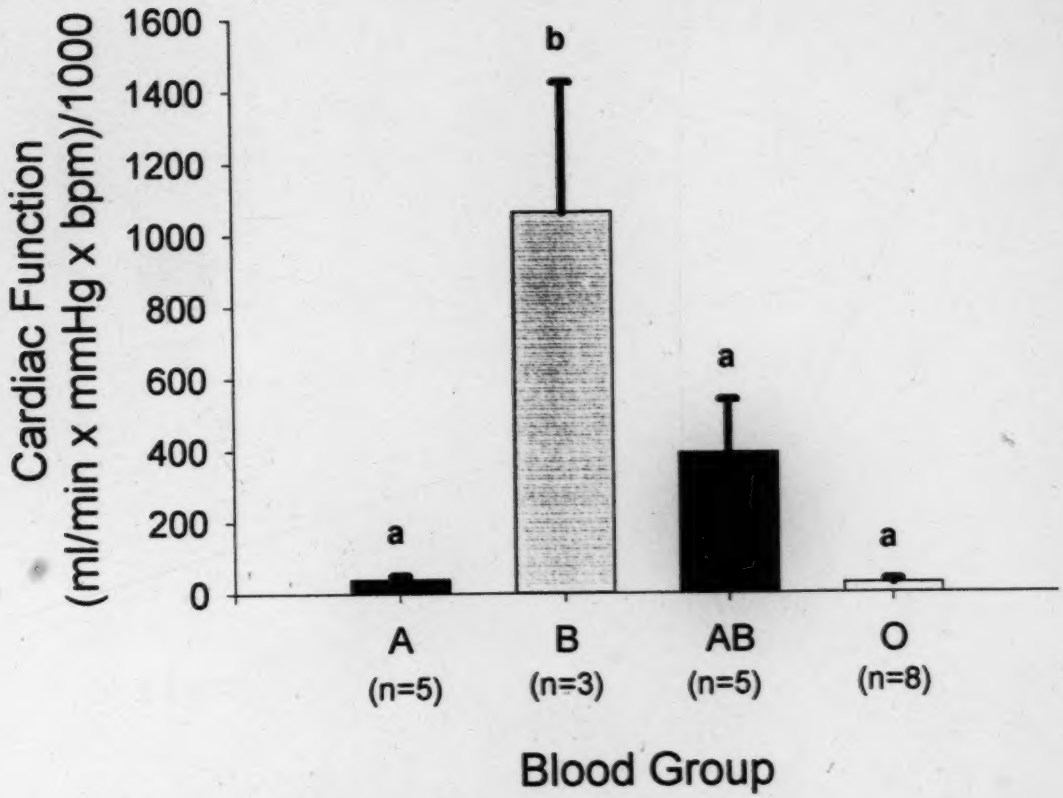


Figure 4.3 Average function of human plasma (15%) perfused pig working hearts over the first 180 minutes based on blood group. Each blood group has a minimum of 5 donors to form the blood group pool. The results are highly significant ($p<0.001$, ANOVA). Post-hoc Tukey tests reveal that only blood group B is statistically different from all other groups. The p-values are: B vs. A, $p<0.001$; B vs. AB, $p=0.015$, and B vs. O, $p<0.001$. The symbols **a** and **b** indicate statistically different groups. Data are expressed as mean ± SEM.

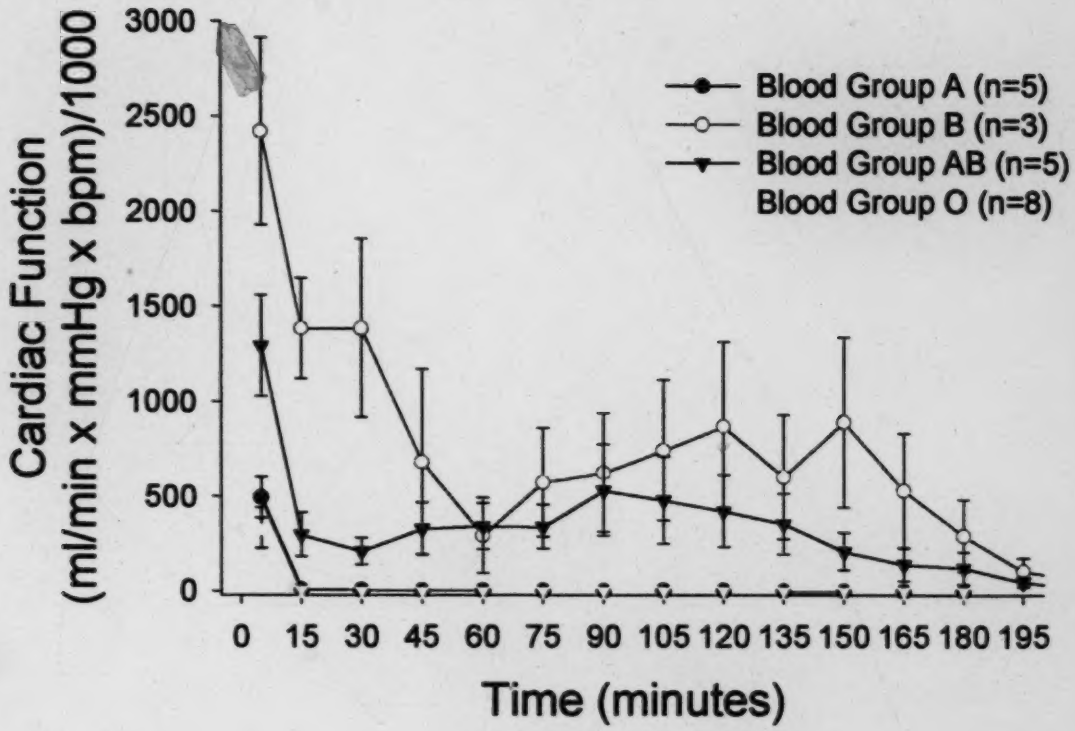


Figure 4.4 Average cardiac function of 15% human plasma perfused pig working hearts over first 195 minutes of perfusion. Blood group B consistently functions better than all other blood groups. Data are expressed as mean ± SEM.

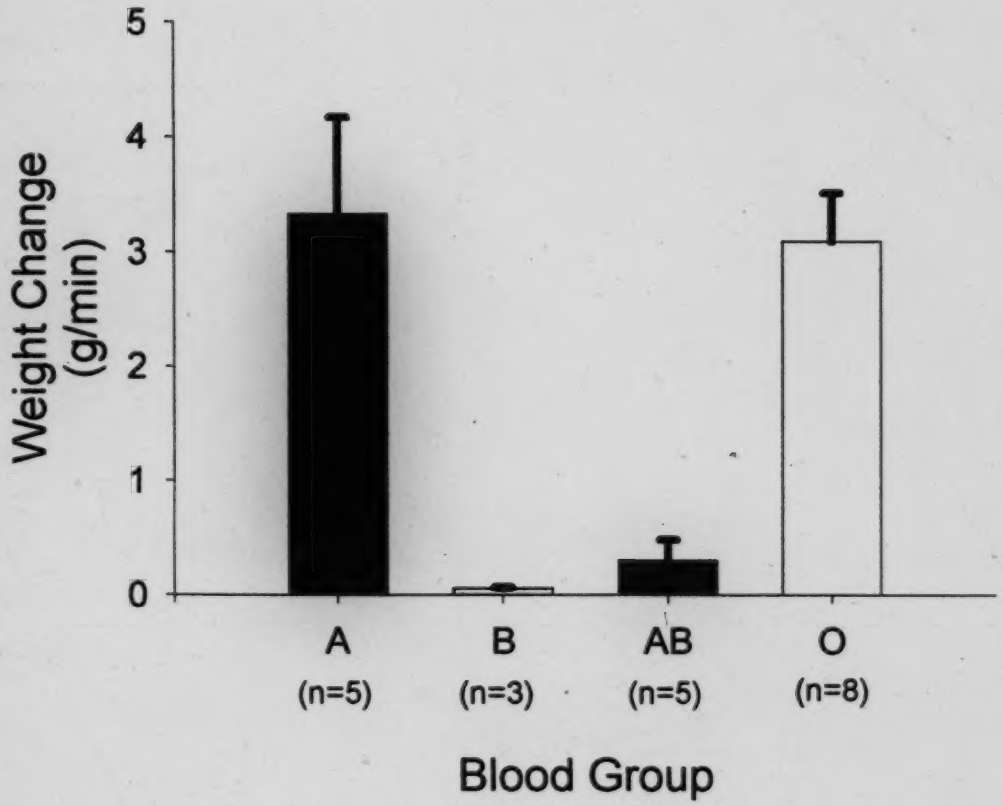


Figure 4.5 Weight gain (edema acculumation) of 15% human plasma perfused pig working hearts. The results are highly signficant (p=0.002, Kruskal-Wallis). Data are expressed as mean ± SEM.

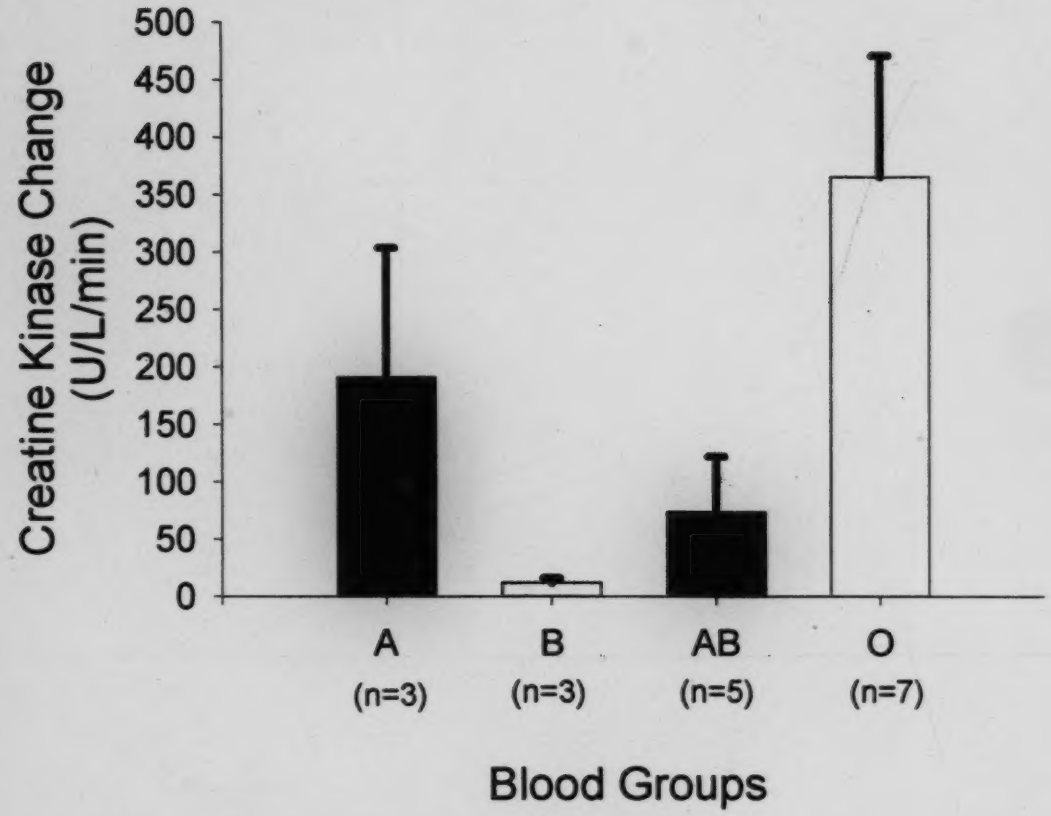


Figure 4.6 Creatine kinase change of 15% human plasma perfused pig working hearts. The results are statistically significantly different (p=0.011, Kuskal-Wallis). Data are expressed as mean ± SEM.

Table 4.3 Comparison of technical factors between human plasma perfused pig working hearts based on blood group

Variable	A (n)	B (n)	AB (n)	O (n)	ANOVA P-value
Total OR time (min)	30 ± 1 (5)	33 ± 2 (3)	33 ± 2 (5)	30 ± 8 (8)	0.315
Total ischemia time (min)	5.4 ± 0.2 (5)	7.0 ± 1.5 (3)	5.8 ± 0.2 (5)	5.6 ± 0.2 (8)	0.205
Total Langendorff Time (min)	13.2 ± 1.4 (5)	13.0 ± 1.0 (3)	13.6 ± 1.1 (5)	20.4 ± 2.9 (8)	0.084
No. of defibrillations	0 ± 0 (5)	0.7 ± 0.3 (3)	0.4 ± 0.2 (5)	0.63 ± 0.3 (8)	0.406
Air in heart (0=no, 1=yes)	0 ± 0 (5)	0 ± 0 (3)	0 ± 0 (5)	0.13 ± 0.13 (8)	0.687
pH	7.58 ± 0.05 (5)	7.40 ± 0.07 (3)	7.37 ± 0.06 (5)	7.53 ± 0.01 (8)	0.08
$[Ca^{2+}]$ (mmol/L)	2.80 ± 0.19 (5)	3.10 ± 0.05 (3)	3.09 ± 0.06 (5)	3.12 ± 0.12 (8)	0.320
Hematocrit (%)	1.4 ± 0.4 (5)	0.9 ± 0.2 (3)	0.9 ± 0.2 (5)	1.0 ± 0.2 (8)	0.478
PO_2 (mmHg)	354.7 ± 28.3 (5)	482.8 ± 8.4 (3)	482.6 ± 22.6 (5)	405.7 ± 0.2 (8)	0.08
$[K^+]$ (mmol/L)	4.27 ± 0.10 [a] (5)	4.69 ± 0.13 [b] (3)	4.65 ± 0.08 [b] (5)	4.28 ± 0.08 [a] (8)	0.001

OR=operation, $[Ca^{2+}]$=calcium concentration, $[K^+]$=potassium concentration
Technical factors of the blood groups were similar except for the average $[K^+]$ where post-hoc Tukey testing showed: B vs. A p=0.037, B vs. AB p=0.989, B vs. O p=0.025. The letters a and b indicate statistically different groups.

Table 4.4 Levels of various factors in human A, B, AB, and O plasma used for pig heart perfusions.

Blood Group	Total IgM (n)	Total IgG (n)	Total IgA (n)	CH50 (n)	α-Gal IgM (n)	α-gal IgG (n)	anti-A antibody (n)	anti-B antibody (n)
A	1.09 ± 0.02 [a] (5)	7.22 ± 0.07 [a] (5)	1.66 ± 0.02 [a] (5)	42.8 ± 0.7 [a] (5)	0.289 ± 0.051 (4)	0.540 ± 0.043 (4)	0 ± 0 [a] (5)	32.00 ± 18.6 [a] (5)
B	0.75 ± 0.03 [b] (3)	8.53 ± 0.36 [b] (3)	1.40 ± 0.04 [b] (3)	37.0 ± 1.5 (3)	0.303 ± 0.012 (3)	0.667 ± 0.057 (3)	53.33 ± 10.67 [b] (3)	0 ± 0 [b] (3)
AB	0.77 ± 0.04 [b] (5)	5.56 ± 0.05 [c] (5)	1.03 ± 0.02 [c] (5)	35.6 ± 1.3 [b] (5)	0.300 ±0.066 (5)	0.596 ± 0.87 (5)	0 ± 0 [a] (5)	0 ± 0 [b] (5)
O	0.89 ± 0.03 [c] (5)	7.95 ± 0.19 [b] (5)	1.87 ± 0.01 [d] (5)	41.6 ± 2.8 (5)	0.267 ± 0.014 (5)	0.822 ± 0.067 (5)	48.00 ± 7.16 [b] (5)	48.00 ± 7.16 [c] (5)
ANOVA	*P<0.001*	*P<0.001*	*P<0.001*	*P=0.0.034*	*P=0.945*	*P=0.051*	*P<0.001*	*P<0.001*

Comparing various factors in the different blood groups reveals a statistically significant difference amongst the blood groups for total IgM, total IgG, total IgA, CH50, anti-A, and anti-B levels. Post-hoc Tukey testing was done to determine the statistically different groups. The symbols a, b, c, and d indicate statistically different groups for the variable being examined. Data are expressed as mean ± SEM.

Table 4.5(a) Univariate linear regression analysis to determine predictors of survival and function of pig working hearts perfused with 15% human plasma.

SURVIVAL REGRESSION			FUNCTION REGRESSION			
Variable	Pearson Correlation Coefficient	P-value	Variable	Pearson Correlation Coefficient	P-value	n
Total IgM	- 0.688	0.002	Total IgM	- 0.524	0.026	18
Total IgG	- 0.102	0.686	Total IgG	0.103	0.686	18
Total IgA	- 0.690	0.002	Total IgA	- 0.438	0.069	18
CH50	- 0.564	0.015	CH50	- 0.535	0.022	18
Anti-α-gal IgM	- 0.030	0.922	Anti-α-gal IgM	0.108	0.726	13
Anti-α-gal IgG	- 0.216	0.478	Anti-α-gal IgG	0.152	0.621	13
Anti-A	0.079	0.755	Anti-A	0.350	0.155	18
Anti-B	- 0.786	<0.001	Anti-B	- 0.609	0.007	18

The blue p-values are statistically significant.

Table 4.5(b) Multivariate linear regression analysis to determine predictors of survival and function of pig working hearts perfused with 15% human plasma.

SURVIVAL REGRESSION			FUNCTION REGRESSION			
Variable	Pearson Correlation Coefficient	P-value	Variable	Pearson Correlation Coefficient	P-value	n
Total IgM	- 0.359	0.06	Total IgM	- 0.226	0.282	18
Total IgA	- 0.022	0.947	CH50	- 0.273	0.277	18
CH50	- 0.164	0.418	Anti-B	- 0.609	0.007	18
Anti-B	- 0.786	<0.001				18

The blue p-values are statistically significant.

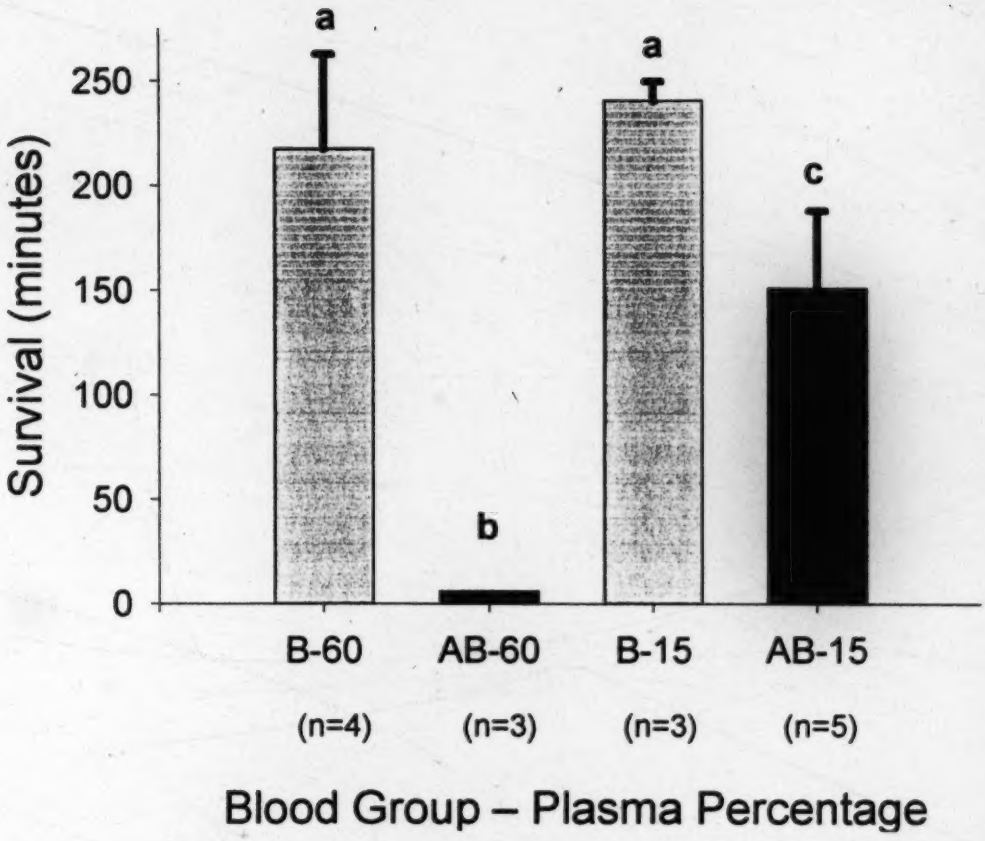


Figure 4.7 Survival of pig working hearts perfused with human B or AB plasma at 60% or 15% concentrations. Groups with different letters (**a**, **b**, or **c**) are significantly different from each other. There are statistically significant differences in survival between blood groups B and AB at both concentrations, however it is much more pronounced at the 60% plasma concentration (15%: $p=0.048$ for B vs. AB and 60%: $p=0.011$ for B vs. AB, t-test). Note also that B-60% is statistically the same as B-15%. Data are expressed as mean ± SEM.

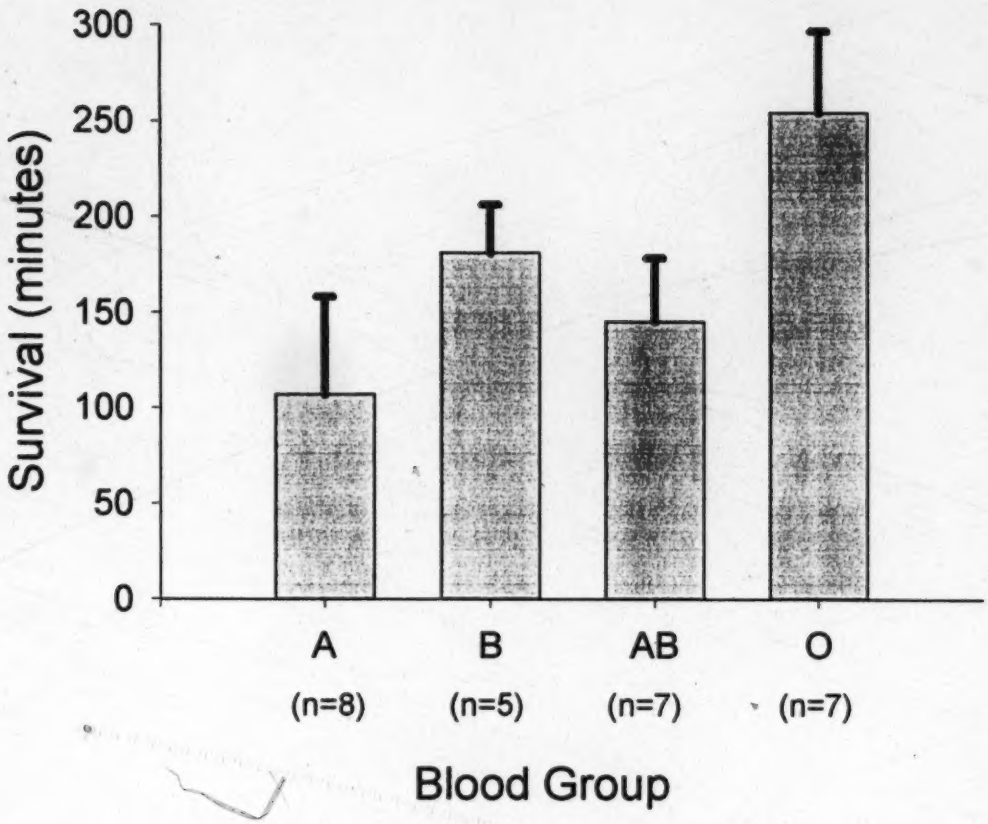


Figure 4.8 Survival of human whole blood pig working hearts based on blood group. There are no significant differences between the groups (p=0.096, ANOVA), however blood group O survives the longest, followed by B, AB, then A. Data are expressed as mean ± SEM.

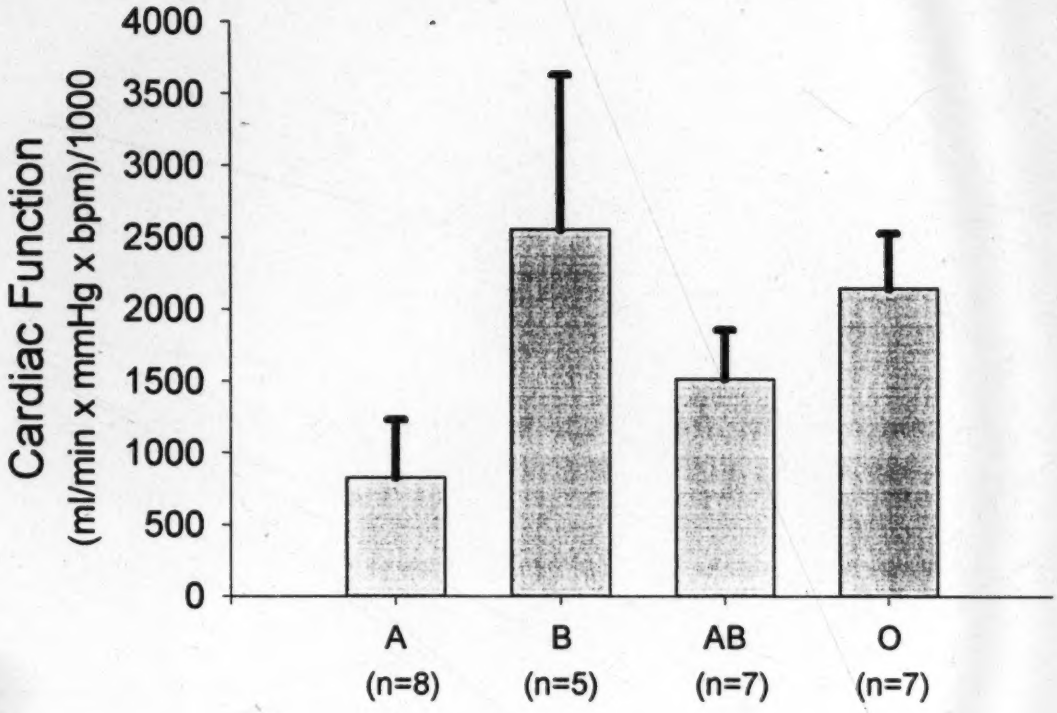


Figure 4.9 Average 180 minute cardiac function of human whole blood perfused working pig hearts based on blood group. There are no significant differences between the groups (p=0.139, ANOVA). In general blood group B has the best function. Data are expressed as mean ± SEM.

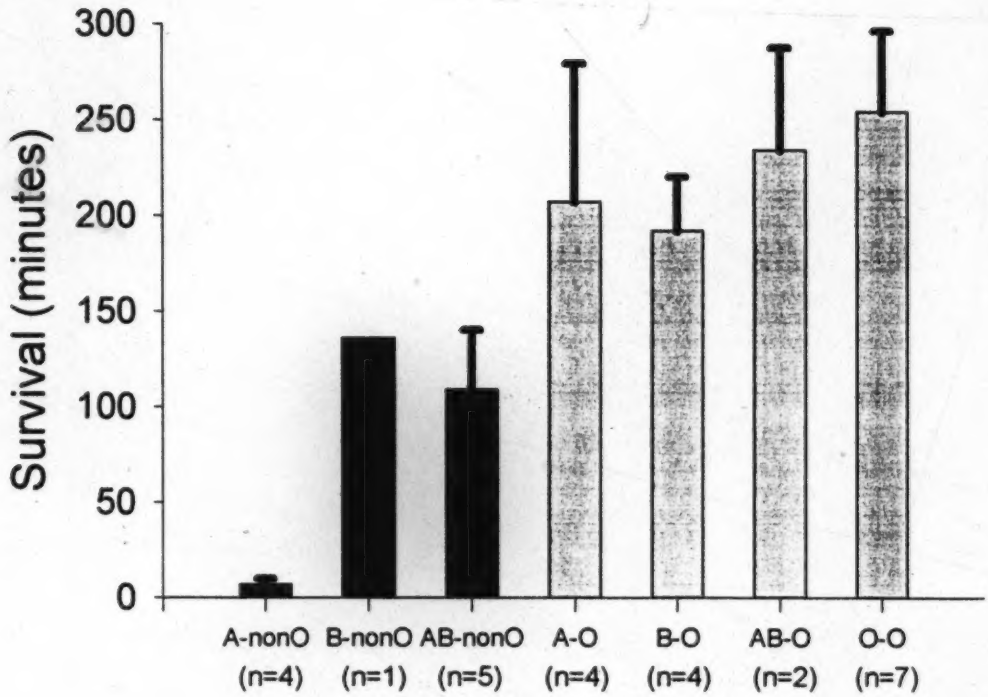


Figure 4.10 Survival of human whole blood perfused pig working hearts based on group. Groups are plasma type A, B, AB, or O and red blood cell type non-O (■) or O (□). Thus, A-nonO means plasma type A and red blood cell type non-O. Since O plasma is only compatible with O red blood cells, there is no "O-nonO" group. There is a statistically significant difference ($p=0.042$, ANOVA) between the non-O groups (■). The O red blood cell groups (□) are not statistically different from one another. If O red blood cells are used, hearts always survive longer. There is a statistically significant difference ($p=0.033$, t-test) between the A-nonO and A-O groups. Data expressed as mean ± SEM.

Table 4.6 Anti-α-gal antibody levels in blood group A whole blood (O RBC versus non-O RBC).

	Anti-α-Gal IgM	Anti-α-Gal IgG	n
O RBC	0.23 ± 0.12	1.22 ± 0.37	4
Non-O RBC	0.54 ± 0.16	1.27 ± 0.20	4
t-test	*P = 0.210*	*P = 0.907*	

Standardized α-gal results. There is no statistically significant difference in the anti-α-gal antibody levels in blood group A based on red blood cell type.

Table 4.7 Anti-α-gal antibody levels in O-whole blood and O-plasma

	Anti-α-Gal IgM	Anti-α-Gal IgG	n
O-Whole Blood	0.24 ± 0.03	0.45 ± 0.12	6
O-Plasma	0.27 ± 0.01	0.82 ± 0.07	5
t-test	*P = 0.634*	*P = 0.082*	

Standardized anti-α-gal antibody ELISA results. There is no statistically significant difference in the anti-α-gal antibody levels to explain the differences seen between O whole blood and O plasma.

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CHAPTER 5

Specific Depletion of Anti-α-Gal Antibody and Human ABO Blood Group in Porcine-to-Human Cardiac Xenotransplantation

Chapter 5: Specific Depletion of Anti-α-Gal Antibody and Human ABO Blood Group in Porcine-Human Cardiac Xenotransplantation

I. Introduction

Investigators have looked at many different ways to prevent hyperacute rejection of pig organs caused by human or non-human primate blood. Some of these have primarily included removal of antibody and complement by organ adsorption, plasmapharesis or non-specfic immunoadsorption columns (1-9). They have also looked at inhibition of antibodies or complement through the use of soluble blockers or depletors and by transgenic means (trying to inhibit complement or prevent α-gal antigen expression) (10-14). Each of these techniques has its advantages and disadvantages. A disadvantage of many of the above techniques is that they over-immunocompromise the recipient with potential subsequent problems of infection and cancer (15). As it is generally accepted that the anti-α-gal antibody is the main antibody of importance in the pig to human combination (16), it would seem reasonable to try to specifically deplete only that antibody (instead of all antibody and complement) and see if that prevents hyperacute rejection.

The Alberta Research Council (Edmonton, Canada) has manufactured an immunoadsorbent - Synsorb 90/ Immunosorb B6 (S90/IB6) which theoretically specifically depletes the anti-α-gal antibody and may have a clinical role. We decided to test this immunoadsorbent using our validated porcine working heart hyperacute rejection model.

II. Objectives

1. To determine the specificity of depletion of S90/IB6 immunadsorbent.
2. To determine whether adsorption of human plasma with S90/IB6 would lead to decreased cytotoxicity against pig aortic endothelial cells (PAEC) when compared to a blank immunoadsorbent (Chromosorb P (CP)), or no immunoadsorbent.
3. To determine whether blood that has been adsorbed with S90/IB6 gives prolongation of *ex-vivo* porcine working cardiac xenografts when compared to blood that has been adsorbed with the blank immunoadsorbent or not immunoadsorbent.
4. To determine if S90/IB6 immunoadsorption effects different human ABO blood groups differently.

We hypothesized that S90/IB6 would specifically deplete human blood of anti-α-gal antibodies and nothing else and this anti-α-gal depleted blood would give less cytotoxicity and longer heart survival (which was not blood group dependent) compared to the blank adsorbed or unadsorbed human blood.

III. Methods:

From previous experiments done by others in the lab, it had been determined that 0.2 grams of S90/IB6 per mL of plasma adsorbed overnight at 4°C gave acceptable

adsorption results (17). Thus, these are the adsorption conditions used for all experiments.

Refer to Chapter 2 for details of other materials and methods used in this chapter.

IV. Results

A) Determining the Specificity of S90/IB6 Immunoadsorption

Table 5.1 shows the change for various factors after adsorption of human plasma with the S90/IB6 immunoadsorbent and the blank (CP) immunoadsorbent. S90/IB6 depletes anti-α-gal antibodies, the B antibody (which is very similar to anti-α-gal antibody) and a small amount of anti-pig aortic endothelial cell (PAEC) IgM antibody subtype.

B) Determining if S90/IB6 Immunoadsorption Decreases Cytotoxicity to Porcine Endothelial Cells

Figure 5.1 demonstrates a statistically significant improvement in survival of pig aortic endothelial cells when S90/IB6 plasma is used compared to unadsorbed plasma or blank (CP) treated plasma.

C) Determining if S90/IB6 Immunoadsorption Leads to Prolongation of Pig Cardiac Xenograft Survival

Figure 5.2 shows the survivals of pig working hearts perfused with whole blood based on the group they belong to (negative control, unadsorbed human, blank (CP) adsorbed, and S90/IB6 adsorbed). The survival times are (mean±SEM): pig negative control (345±17 min.), unadsorbed human (169±23 min.), blank adsorbed (131±27 min.), and S90/IB6 adsorbed (262±21 min.). As can be seen, the S90/IB6 group is significantly

better ($p<0.001$) than the unadsorbed human group, and the blank (CP) adsorbed group; however, it is statistically similar to the negative control group. **Figure 5.3** demonstrates cardiac function of the hearts. Here, S90/IB6 is not statistically better than the unadsorbed or blank adsorbed groups, nor is it statistically worse than the pig blood group. The trend is for S90/IB6 to have better cardiac function. **Figure 5.4** shows a statistically significant difference ($p<0.001$) for weight change (edema accumulation) again with S90/IB6 appearing to be similar to pig and different from unadsorbed or blank adsorbed blood. **Figure 5.5** shows that there are no statistically significant differences between the groups for creatine kinase change although there are obvious absolute differences between the groups.

It appears from these results that immunoadsorption with S90/IB6 had a significant impact. To confirm that the results were not due to different technical factors between the groups, these factors were examined (**Table 5.2**). As can be seen, the statistically significant differences are between pig and other groups and not between S90/IB6 and other groups. The S90/IB6 group is similar to the unadsorbed human and blank adsorbed groups. Thus, the differences observed in survival with the S90/IB6 is not a technical bias towards one or another group.

From previous work, it has been shown that human red blood cells can have variable results on the hearts and that O-red blood cells tend to be protective. Therefore, the distribution of human O-red blood cells and human non-O-red blood cells in the S90/IB6 group was examined. Survival of S90/IB6 whole blood hearts having O red blood cells ($n=7$) was 296 ± 22 minutes versus non O red blood cells ($n=6$) which was 221 ± 31 minutes ($p=0.067$, t-test). Thus, S90/IB6 gave prolonged survival regardless of

blood type. The above result although not significant, was close to being significant. Because of this, and because early experiments had shown that human red blood cells can have variable effects, the S90/IB6 was tested just using plasma. **Figure 5.6** shows survival of pig, unadsorbed human and S90/IB6 adsorbed plasma-only perfused hearts. Once again that S90/IB6 is similar to pig and different from unadsorbed human. Cardiac function (data not shown) of the S90/IB6 hearts is significantly better ($p<0.001$, post-hoc Tukey) than than unadsorbed human and both creatine kinase (CK) change and weight change are significantly different for S90/IB6 compared to unadsorbed human. The CK changes (in U/L/min) of the groups (mean±SEM) are: pig (22.9±3.2, n=2), unadsorbed human (196.4±56.2, n=18) and S90/IB6 (18.6±1.9, n=12) – $p=0.037$ (ANOVA). Post-hoc Tukey showed pig vs. unadosrbed human ($p=0.03$), pig vs. S90/IB6 ($p=0.999$), and unadsorbed vs. S90/IB6 ($p=0.027$). For weight change (edema accumulation) in grams/min., the values are (mean±SEM): pig (0.11±0.02, n=2), unadsorbed human (2.05±0.40, n=21) and S90/IB6 (0.10±0.01, n=12) – $p=0.002$ (ANOVA). Post-hoc Tukey testing again revealed pig vs. unadsorbed ($p=0.003$), pig vs. S90/IB6 ($p=1.00$) and unadsorbed vs. S90/IB6 ($p=0.002$). S90/IB6 therefore is significantly better than unadsorbed human plasma and statistically similar to the autologous pig plasma group. Thus, S90/IB6 adsorption of human blood prolongs survival.

Although it appeared that S90/IB6 immunoadsorption prevented hyperacute rejection, when light microscopy (**Figure 5.7**) and immunofluorescence (**Figure 5.8**) of the whole blood perfused hearts was examined, there was evidence of hyperacute rejection and antibody/complement binding on the S90/IB6 hearts. **Figures 5.9** and **5.10**

graphically demonstrate that S90/IB6 histologically and via immunofluorescence resembled the unadsorbed human and blank (CP) groups.

C) Determining if S90/IB6 Adsorption Effects Different Blood Groups Differently

Figure 5.11 shows the effect of S90/IB6 adsorption on survival via blood group for hearts perfused with 15% human plasma. **Table 5.3** examines the effect of S90/IB6 adsorption on function, creatine kinase change, and weight change via blood group for hearts perfused with 15% human plasma. From the data, only blood groups A and O improve significantly in terms of survival, function and weight change. Blood group O also improves in terms of creatine kinase change. Blood group AB improves in function with S90/IB6 treatment; however, blood group B plasma does not benefit at all by S90/IB6 immunoadsorption.

Figure 5.12 and **Table 5.4** examine the benefits of S90/IB6 immunoadsorption for whole blood perfused hearts. Blood group A significantly benefits in survival from treatment with S90/IB6. Though the other measures are not statistically significant, there are large improvements in function, creatine kinase change and weight change for blood group A. Thus, S90/IB6 is beneficial for blood group A. Blood group B also has some benefit with S90/IB6 but this group is difficult to analyze secondary to small sample size in B-S90/IB6. Blood group AB statistically improved only in function with S90/IB6 treatment; however, if looking at absolute numbers, blood group AB did improve in all parameters with S90/IB6 treatment. Finally blood group O did improve in all parameters (except for function) with S90/IB6 adsorption; however, only weight change was statistically significant. These observations will be examined in the discussion.

A closer look at the unadsorbed human blood group hearts perfused with O red blood cells (regardless of actual blood group, n=17) and all whole blood S90/IB6 (n=13) hearts (regardless of blood group or red cell type) **(Table 5.5)** reveals that survival, function and other parameters are statistically similar between the two. This suggests that if one is using O red blood cells, then one may not need S90/IB6 immunoadsorption or may not need as much immunoadsorption as the O red blood cells seem to offer some protection.

V. Discussion

The organ shortage crisis in allotransplantation may be solved by the use of pig organs (15). Numerous studies have shown the importance of the anti-α-gal antibody in causing hyperacute rejection in the pig-to-human combination (18-23). However, a potentially clinically applicable anti-α-gal immunoadsorbent has never been tested in a pig working heart model perfused with human blood. S90/IB6 immunoadsorbent is a compound that was purchased from the Alberta Research Council, and this compound has been shown to deplete anti-α-gal antibody (17). However, the specificity of this compound and the use of this compound with pig working hearts have never been tested before. We used our validated porcine-human working heart model of xenotransplantation to test the S90/IB6 immunoadsorbent.

A) S90/IB6 Specifically Depletes Anti-α-gal and Anti-α-Gal Like Antibodies

Anti-α-gal antibodies recognize the following antigens: gal α1,3 gal disaccharide, gal α1,3 gal β1,4 glc trisaccharide, gal α1,3 gal β1,4 GlcNAc trisaccharide and other

tetra- and pentasaccharides that all have gal α1,3 gal at the terminal end (16,18). The more terminal the sugar (ie: closer to the outside where antibody can see it), the more important the sugar is in antibody binding (16). In fact, the only difference between individuals who are blood group A and B is the terminal sugar. Reaction to this sugar leads to very severe rejection (24). **Table 5.1** demonstrates that S90/IB6 does not remove all antibody and complement which other methods of anti-α-gal depletion do (e.g. Therasorb-Ig, plasmapharesis and organ adsorption) (1,25). S90/IB6 depletes anti-α-gal antibody as measured by our anti-α-gal specific ELISA. S90/IB6 also depletes the anti-B antibody as measured by a hemagglutination assay. As this latter assay is less accurate, S90/IB6 likely does not deplete anti-B better than anti-α-gal, but likely depletes it to the same extent. S90/IB6 is still considered to be an anti-α-gal immunoadsorbent as anti-B has gal α1,3 gal in its terminal positions. The fact that S90/IB6 depletes anti-B antibody gives additional evidence to support conclusions from the last chapter that the mechanism involved with blood group differences is the anti-B antibody cross reacting with the α-gal antigen augmenting rejection. Immunoadsorption conditions are less stringent than ELISA conditions and that is why anti-B antibody can cross react with α-gal antigens in immunoadsorption, but not in ELISA assays. One may question whether it is the anti-α-gal antibody or the anti-B antibody which is important in causing hyperacute rejection in our model as it was only blood groups A and O plasma (both have anti-B) that improved significantly in survival after treatment with S90/IB6. The anti-α-gal antibody is involved and important because all of the blood groups (perfused with whole blood, and all, except B, perfused with plasma) improved in survival after S90/IB6 treatment. Blood group AB (does not have anti-A nor anti-B) had large (though not statistically significant)

improvements in survival and it did have statistically significant improvements in function after S90/IB6 treatment. Others (17,18,20) using blood group AB have found improvements after removing or inhibiting the anti-α-gal antibody. Thus, the anti-α-gal antibody is important; however, the presence of anti-B likely augments the rejection process. S90/IB6 also depletes a small percentage of anti-PAEC IgM antibody. This would be expected as most α-gal antigen is present on endothelial cells. Specific depletion of factors such as coagulation proteins or cytokines were not examined, however, we do know that the plasma was well anticoagulated (PTINR >13.2, PTT>200) before adsorption and these numbers did not change post adsorption.

B) Anti-α-gal Depletion Prolongs Xenograft Survival

Testing our anti-α-gal specific (S90/IB6) immunoadsorbant revealed that it gave longer survival, less heart muscle damage (as measured by creatine kinase change) and less edema accumulation (measured by weight change) compared to the unadsorbed human blood and the blank (CP) adsorbed human blood when whole blood was tested (**Figures 5.1-5.5**). Thus (S90/IB6) specfically depletes α-gal antibody and this leads to prolongation of xenograft survival.

When whole blood was used, S90/IB6 gave function similar to pig blood as well as unadsorbed human blood or blank adsorbed blood. S90/IB6 function was better than blank adsorbed or unadsorbed human blood but not statistically different. However, when plasma-only was examined, function of S90/IB6 hearts was significantly better than function of unadsorbed human plasma. The latter (plasma-only) finding is what was expected. The former (whole blood) results may be so because red blood cells may have

given enough benefit to the other groups to have statistical significance lost in our system.

There are many novel findings from our work compared to that existing in the literature. Aspeslet et al. (17) have shown the efficacy of (S90/IB6) immunoadsorbant *in vitro* (cytotoxicity assays). Xu *et al.* (26) have used this immunoadsorbant in pig-to-baboon kidney xenotransplants and found the immunoadsorbant to be effective in prolonging pig kidney xenograft survival compared to unadsorbed baboon blood. A significant and novel aspect of our study is the finding that the immunoadsorbant is effective in an organ other than the kidney – namely the porcine heart. This is an important finding because the heart is a more sensitive organ to injury, and thus needs to be investigated separately. There are many reasons that the heart is a more sensitive organ to detect injury. Kidneys need to maintain various cellular pumps to perform their functions and this requires energy. Hearts not only have to maintain cellular pumps, but in addition, have to generate mechanical force and do work to pump blood. Other evidence suggesting the heart is more sensitive to injury can be seen in the fact that clinical heart discordant xenotransplants done in humans generally survived less than a day where as kidneys survived for a few days at least (Chapter 1). Lastly, in allotransplantation, hearts in general cannot tolerate as long an ischemia time period as kidneys can. For the above reasons, the heart is more sensitive to injury and thus needs to be specifically tested (finding that the immunoadsorbent is effective in kidneys does not mean that it will be effective with hearts). Another novel finding of our study is that we used human blood in our system. *In vivo* experiments in some non-human primates may not accurately reflect what will happen in a human as some non-human primate blood is

different from human blood (15,27,28). Examples of the differences between human blood and baboon blood include the fact that baboon blood types generally are 1/6-1/3 A, 1/3 AB, and 1/3 B (with very rare baboons being blood type O). Even these blood types don't type standardly with human reagents suggesting differences in human/baboon blood types. Blood group differences are very important in our project. As our model uses human blood, a more direct extrapolation to *in vivo* human trials can be made.

There have been *ex-vivo* porcine working heart experiments using human blood as well as *in vivo* pig-to-baboon orthotopic heart transplant experiments done using an immunoadsorbant called Therasorb-Ig (25). As mentioned in chapter 1, Therasorb-Ig nonspecifically removes large amounts of total IgM, total IgG, and total complement (8,9,25). Nonspecifically removing all of these immunological components is not desirable as it leaves the recipient more prone to infectious complications (15). S90/IB6 immunosorbant specifically removes anti-α-gal antibodies and anti-α-gal like (anti-B) antibodies, not total IgM, IgG, IgA, IgE, complement, or protein and this specific removal leads to prolongation of xenogrft survival – a novel finding.

Use of S90/IB6 may be better than other methods of addressing hyperacute rejection. Other researchers have looked at complement inhibition (using soluble complement receptor type I, cobra venom factor, or transgenic pigs expressing human complement regulatory proteins) to prevent hyperacute rejection (10-14). Our method (immunoadsorption with anti-α-gal specific columns) of preventing hyperacute rejection is a better alternative than soluble complement receptor type I or cobra venom factor due to less expense of the column, potential reusability of the column and less toxicity (as may occur with CVF). CVF actually has an α-gal structure in it (10) and has been found

to induce production of anti-α-gal antibodies (15). Use of transgenic pigs will likely be complementary to use of the anti-α-gal immunoadsorbant. The advantage of using combination therapy is that the immune system is very redundant, thus, having many strategies targetting the immune system will be desirable (15,16). Use of the immunoadsorbent column will be important with the transgenic pigs as these pigs still undergo antibody mediated rejection later on and some of the antibodies are anti-α-gal antibody (15).

There are problems with the use of the anti-α-gal specific immunoadsorbent. Earlier studies that we did in cynomologous monkeys showed that even though one could get adequate depletion of the anti-α-gal antibody with the (S90/IB6) column, the anti-α-gal antibody rebounded back to baseline levels by ~24hrs and went even higher thereafter (Latham, Manji, unpublished). Similar findings were obtained by Cooper *et al.* (29), who actually found that even with repeated immunoadsorptions using S90/IB6, rebound still occurred, though not as quickly and not as high with latter immunoadsorptions. This rebound phenomenon may be related to sensitization of B cells that either pass by the antigen on the column during immunoadsorption or by antigen that travels into the monkey thereby stimulating the B cells *in vivo.* We (Manji unpublished) did a small study to see if immunosuppressive drugs known to have activity against B cells would suppress this rebound. In one monkey, the anti-α-gal IgG was permanently suppressed and in another we could no longer absorb out any anti-α-gal antibody. The significance of these findings is not clear, as the numbers are small. Thus, the anti-α-gal immunoadsorbent is beneficial but it will need to be used with other immune therapy. Another problem associated with anti-α-gal depletion is what the consequences of having

low anti-α-gal antibody may mean to humans. As discussed in Chapter 1, anti-α-gal antibody is believed to be involved with the elimination of senescent red blood cells (30-34) as well as protection against pathogens and tumors (35,36). It is possible that humans may have problems with the above if there is no anti-α-gal antibody present. However, there are individuals with low anti-α-gal antibody who are surviving. Perhaps we will just need to be more aware and be proactive in addressing any problems that may occur with anti-α-gal antibody depletion.

The other problem associated with the S90/IB6 immunosorbent is that passing whole blood through the columns (as was done with monkey blood) lead to hemolysis (anemia), leukopenia and thrombocytopenia (Manji, unpublished). The potential ways to solve some of these problems may be to use a plasmaphereis machine with very secure housing for the immunoadsorbent beads such that only plasma (no cells such as B cells or red blood cells/platelets) would pass through the immunoadsorbent hopefully eliminating the hemolysis, leukopenia, thrombocytopenia, and sensitization problems. The anti-α-gal specific columns however are effective and though some refinements and adjuncts are needed, this technology should prove very useful in clinical practice.

Even though S90/IB6 prevented hyperacute rejection by many of the parameters measured, pathologic examination actually showed evidence of hyperacute rejection (**Figures 5.7-5.10**). The S90/IB6 hearts had similar amounts of hemorrhage, thrombosis, antibody, and complement staining as the unadsorbed human and blank (CP) adsorbed hearts. In addition, if a Dunnett's post-hoc statistical test was done (as opposed to a Tukey test which was done), the S90/IB6 hearts were statistically worse ($p=0.04$) compared to pig blood though they were still statistically better than the unadsorbed or blank(CP)

groups. All of the above suggests that other immunological factors are involved besides just anti-α-gal antibody and again emphasizes the importance of combination immune therapy for patients. Investigators found that α-gal knockout mice still underwent hyperacute rejection when perfused with human sera. **Table 5.6** lists other antigens against which humans can have naturally occurring antibodies. Pigs express 10-30% of these antigens (16). These antibodies may be the important "other immunological factors" discussed above.

C) S90/IB6 Immunoadsorption Effects Human ABO Blood Groups Differently:

Immunoadsorption of anti-α-gal antibody did not lead to prolonged graft survival in all the blood groups. Only blood group A and O plasma perfused hearts had a significant improvement in survival after depletion of anti-α-gal antibody (**Figure 5.11**). However, blood group A, AB and O had a significant improvement in function and had less edema accumulation and less creatine kinase rise after anti-α-gal depletion (though the latter two were not always significant). It is possible that had one tested S90/IB6 in 60% AB plasma hearts, one may have found a statistically significant difference in survival for blood group AB (as the AB-60% plasma hearts only survived 6 minutes). However, it is less likely that one would have found a similar benefit for blood group B as B-60% survived 220 minutes which was the same as S90/IB6 B-15% plasma group of 226 minutes.

When whole blood perfused hearts were examined, all the groups had an improvement in survival after S90/IB6 immunoadsorption but only blood group A had a statistically significant improvement (**Figures 5.12**). Blood group AB had a statistically significant improvement in function (similar to plasma-only AB hearts) and blood group

O had a statistically significant improvement in weight change (similar to plamsa-only O hearts, **Table 5.4**). The major point of note was that blood group O did not have a significant improvement in survival with S90/IB6 (which is very different from the plasma-only O hearts). The possible reasons that blood group O did not improve significantly with S90/IB6 may be just that the unadsorbed O group did so well and the working heart model system had an upper limit so that the O-S90/IB6 group could not be kept functioning for many more hours to see if one could get statistical significance. It is possible that one may have actually found a statistically significant difference between O-unadsorbed and O-S90/IB6 had the O-S90/IB6 hearts been able to work longer. This is suggested by the fact that the CK change is less in the O-S90/IB6 group and the weight change is significantly less in the O-S90/IB6 group. The function of the O-S90/IB6 group was not very good though this was not statistically significant. The reason for the poorer function is not clear.

The above reinforce the importance of anti-α-gal antibody depletion in porcine-human xenotransplantation, but suggest that perhaps certain blood groups (AB and more so B) may not need as much immunoadsorption to achieve the same level of benefit. This would need to be tested using smaller amounts of immunoadsorbant and/or less immunoadsorption time in blood groups B and AB to see if overall less immunoadsorption still gave adequate improvement in function/edema/CK rise as the higher amounts of immunoadsorption. This would be very important to know clinically as it may mean that certain blood groups would not need as much immunoadsorption and thus less costs and problems associated with immunoadsorption than people who are blood group A (or O). To our knowledge these findings are novel as no one has

investigated the differential effects of human ABO blood group in porcine heart survival after specific anti-α-gal immunoadsorption.

As whole blood group O-unadsorbed hearts functioned so well (especially comparing them to O plasma-only hearts); and as it is known from previous chapters that O red blood cells may be protective, examination of all unadsorbed whole blood perfused hearts (regardless of plasma blood group) which had O red blood cells was done and compared to all 13 S90/IB6 whole blood hearts. **Table 5.5** suggests that if O red blood cells are used, statistically, one obtains similar results to S90/IB6 immunoadsorption, though for absolute numbers, the S90/IB6 group is better. Clinically, this suggests that transfusing O red blood cells may allow less immunoadsorption need and thus less problems associated with immunoadsorption (such as cost, practicality, etc.). The possible reasons for O red cell benefit are discussed in chapter 3.

Overall, S90/IB6 is an effective immunoadsorbent. Specific depletion of anti-α-gal and anti-α-gal like antibodies leads to prolongation of xenograft survival. Clinically, one will need to treat individuals differently based on blood group. O red blood cells may be a useful adjunct.

VI. Figures and Tables

Table 5.1 Percent change of various factors from human plasma after immunoadsorption with S90/IB6 or Blank (CP) immunoadsorbent.

Variable	S90/IB6 (n)	Blank-CP (n)
Total IgM	-1.90 ± 0.20 (10)	-1.67 ± 0.55 (8)
Total IgG	-0.40 ± 0.44 (10)	-0.30 ± 0.84 (8)
Total IgA	0.42 ± 0.78 (10)	1.11 ± 0.68 (5)
Total IgE	-0.23 ± 0.85 (6)	1.44 ± 1.44 (4)
CH50	-3.83 ± 3.69 (12)	1.63 ± 3.81 (6)
Anti-α-gal IgM	-84.70 ± 6.5 (13)	-6.40 ± 6.20 (12)
Anti-α-gal IgG	-80.00 ± 4.0 (13)	-3.40 ± 12.60 (12)
Anti-PAEC IgM	-10.40 ± 3.60 (13)	1.60 ± 9.10 (12)
Anti-PAEC IgG	- 1.40 ± 3.10 (13)	-6.50 ± 6.50 (12)
Anti-A	-16.67 ± 27.13 (6)	-25.00 ± 25 (2)
Anti-B	-91.67 ± 1.32 (6)	-10.00 ± 24.49 (6)
Total Protein	-0.29 ± 0.19 (10)	0.50 ± 0.40 (8)
Cholesterol	1.48 ± 0.36 (10)	2.90 ± 1.21 (4)
Triglycerides	3.86 ± 1.80 (10)	1.78 ± 0.65 (3)

S90/IB6 specifically depletes anti-α-gal and anti-α-gal like (anti-B) antibody.
The anti-B and anti-A antibody depletions were determined by hemagglutination assay which are not as accurate as anti-α-gal ELISA assays.
CH50 is a measure of complement activity.
PAEC=pig aortic endothelial cells

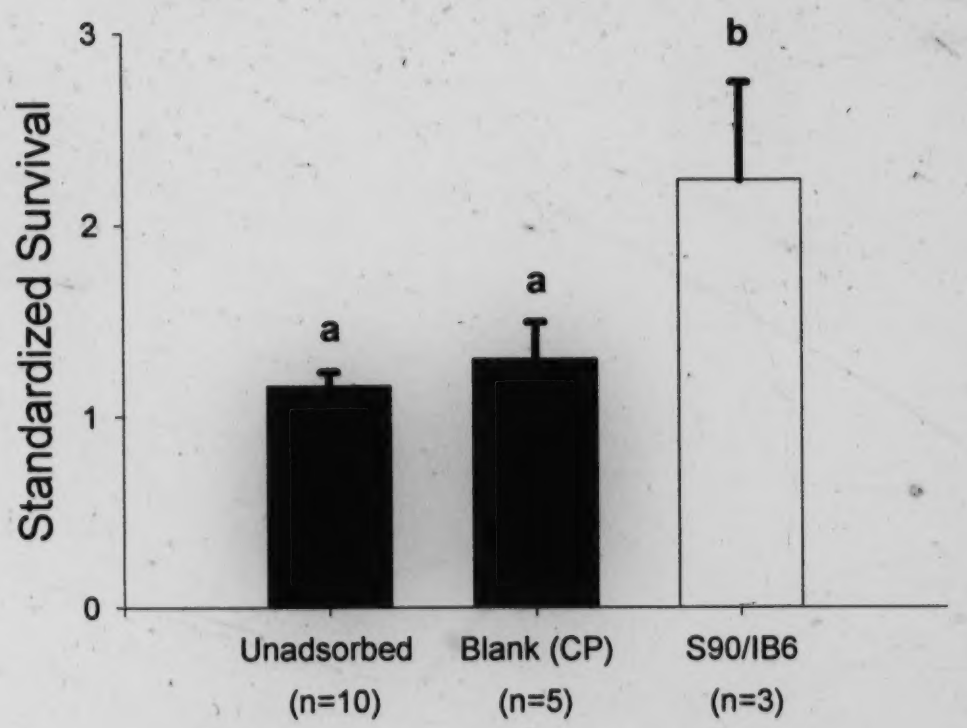

Figure 5.1 Survival of pig aortic endothelial cells in MTT cytotoxicity assay comparing unadsorbed, blank adsorbed (CP), or S90/IB6 adsorbed human plasma. Survivals are standardized against a control serum to allow comparison between different plates. Anti-α-gal depletion significantly improves survival ($p=0.017$, ANOVA). Post-hoc Tukey's testing demonstrated S90/IB6 to be significantly different from unadsorbed or blank (CP) groups. The symbols **a** and **b** denote statistically different groups. Data are expressed as mean ± SEM.

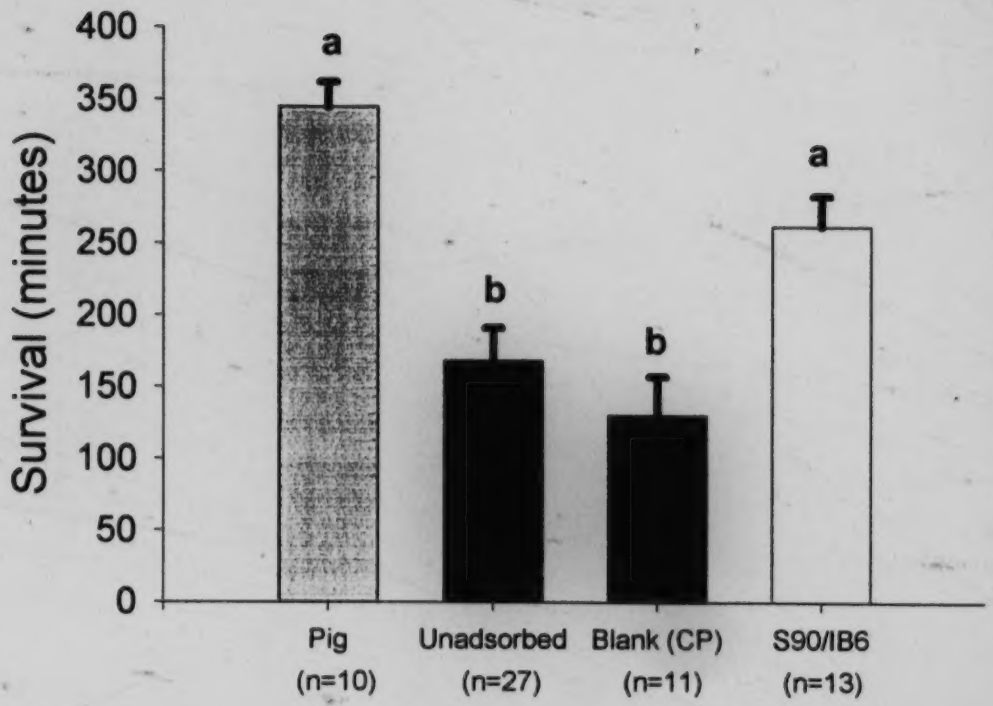

Figure 5.2 Survival of pig working hearts perfused with pig whole blood, unadsorbed, blank (CP) adsorbed or S90/IB6 adsorbed human whole blood. There is a statistically significant difference ($p<0.001$, ANOVA) between the groups. Post-hoc Tukey testing shows S90/IB6 and pig to be similar to one another and different from unadsorbed and blank (CP) groups. The symbols **a** and **b** denote statistically different groups. Anti-α-gal antibody depletion prolongs xenograft survival. Data are expressed as mean ± SEM.

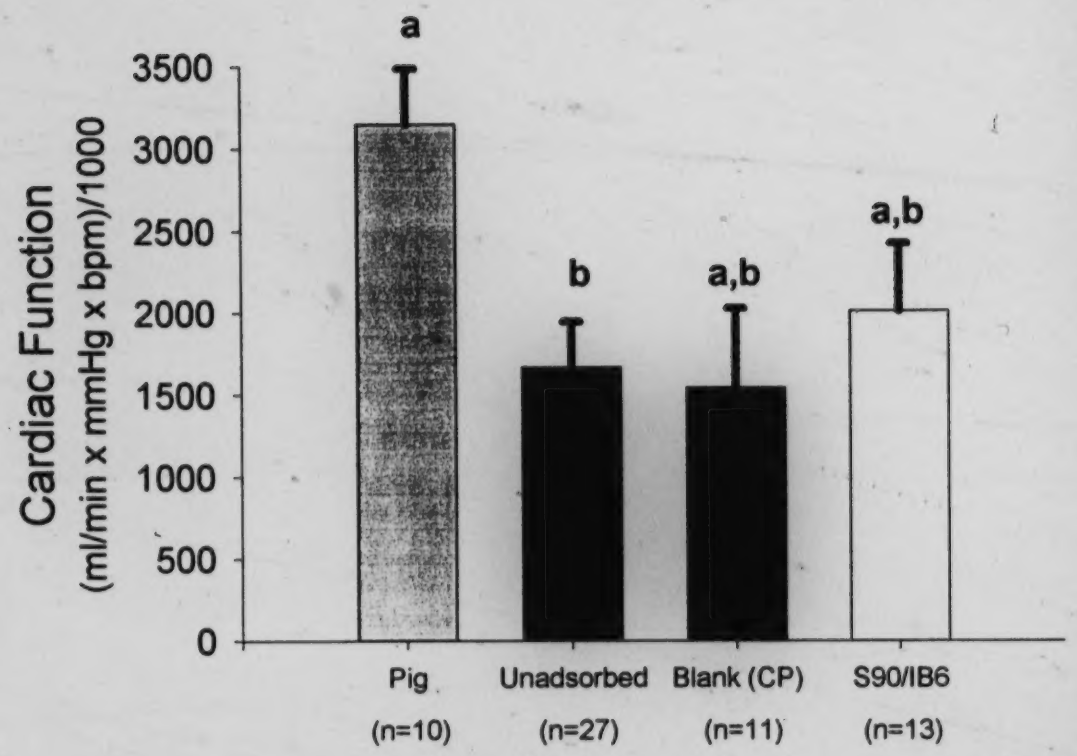

Figure 5.3 Average function of pig working hearts over the first 180 minutes of perfusion by group. Groups are pig whole blood, unadsorbed, blank (CP) adsorbed, or S90/IB6 adsorbed human whole blood. There is a statistically significant difference ($p=0.038$, ANOVA) between the groups. Post-hoc Tukey testing shows pig blood to be different from unadsorbed human blood. The blank and S90/IB6 groups are statistically similar to both unadsorbed human blood and pig blood. The symbols **a** and **b** denote statistically different groups. Data are expressed as mean ± SEM.

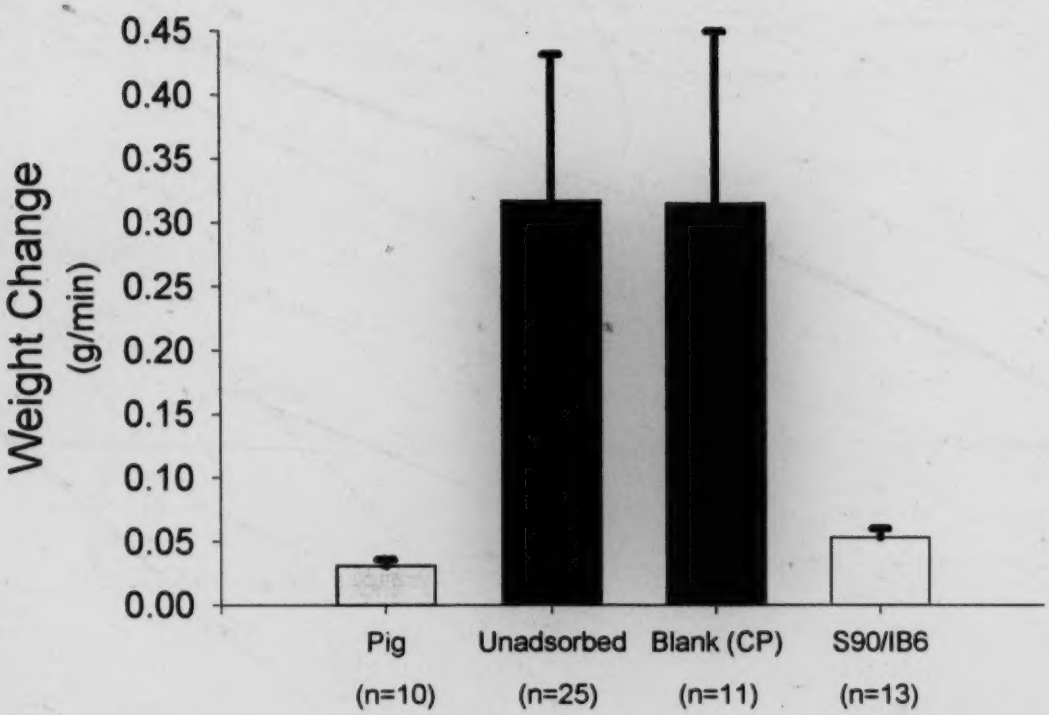

Figure 5.4 Weight change (edema accumulation) of pig working hearts perfused with pig whole blood, unadsorbed, blank (CP), adsorbed, or S90/IB6 adsorbed human whole blood. There is a statistically significant difference between the groups ($p<0.001$, Kruskal-Wallis). Data are expressed as mean ± SEM.

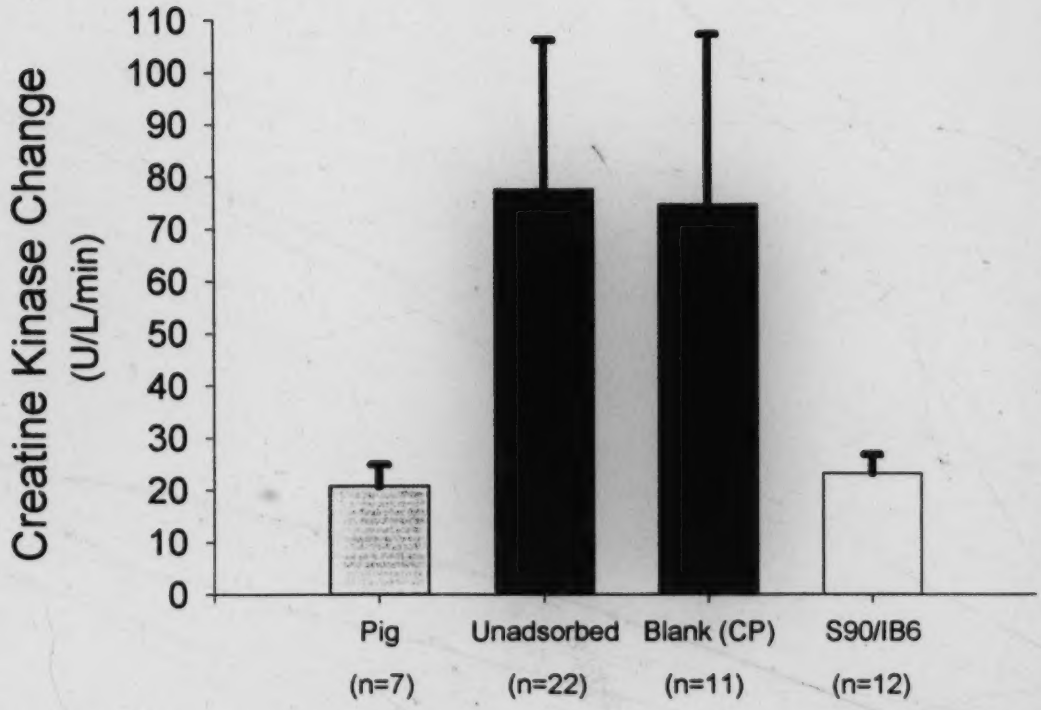

Figure 5.5 Creatine kinase changes of pig working hearts perfused with pig whole blood, unadsorbed, Blank (CP) adsorbed, or S90/IB6 adsorbed human whole blood. There are large absolute differences between the groups with S90/IB6 being similar to pig and being different from unadsorbed and blank adsorbed human blood ($p=0.06$, Kruskal-Wallis). Data are expressed as mean ± SEM.

Table 5.2 Comparison of technical factors between pig whole blood, unadsorbed, blank (CP) adsorbed, or S90/IB6 adsorbed human whole blood perfusing pig working hearts.

Variable	Pig (n)	Unadsorbed Human (n)	Blank (CP) (n)	S90/IB6 (n)	P-Value (ANOVA)
Total OR time (min)	61 ± 5 (10)	30 ± 1 (27)	31 ± 11 (11)	30 ± 1 (13)	<0.001
Total ischemia time (min)	8.6 ± 1.0 (10)	6.4 ± 0.6 (27)	6.5 ±0.4 (11)	7.21 ± 1.3 (13)	0.331
Total Langendorff Time (min)	26.9 ± 4.0 (10)	20.7 ± 0.9 (27)	21.3 ± 1.2 (11)	19.2 ± 1.1 (13)	0.044
No. of defibrillations	0.6 ± 0.2 (10)	0.2 ± 0.1 (27)	0.4 ± 0.1 (11)	0.3 ± 0.2 (13)	0.284
Air in heart (0=no, 1=yes)	0.2 ± 0.1 (10)	0.03 ± 0.03 (27)	0 ± 0 (11)	0.08 ± 0.08 (13)	0.212
pH	7.40 ± 0.01 (7)	7.44 ± 0.02 (27)	7.44 ± 0.02 (11)	7.44 ± 0.0 (13)	0.700
$[Ca^{2+}]$ (mmol/L)	3.21 ± 0.13 (9)	3.20 ± 0.07 (27)	3.37 ± 0.14 (11)	3.29 ± 0.13 (13)	0.694
Hematocrit (%)	13.2 ± 1.2 (9)	16.2 ± 0.5 (27)	17.1 ± 0.6 (11)	15.9 ± 0.5 (12)	0.009
PO_2 (mmHg)	429.1 ± 24.8 (8)	469.4 ± 13.2 (26)	462.6 ± 32.4 (11)	490.6 ± 21.8 (12)	0.441
$[K^+]$ (mmol/L)	6.0 ± 0.4 (7)	4.83 ± 0.2 (26)	4.7 ±0.2 (11)	4.8 ± 0.3 (12)	0.009

OR=operation, $[Ca^{2+}]$=calcium concentration, $[K^+]$=potassium concentration
Post hoc Tukey tests showed the following: for OR time, the pig group was significantly different from all other groups; for Langendorff time, the pig group was significantly different from the S90/IB6 group; for hematocrit, pig was significantly different from unadsorbed and blank adsorbed blood but not S90/IB6; and for potassium, pig was significantly different from all other groups.
Data are expressed as mean ± SEM.

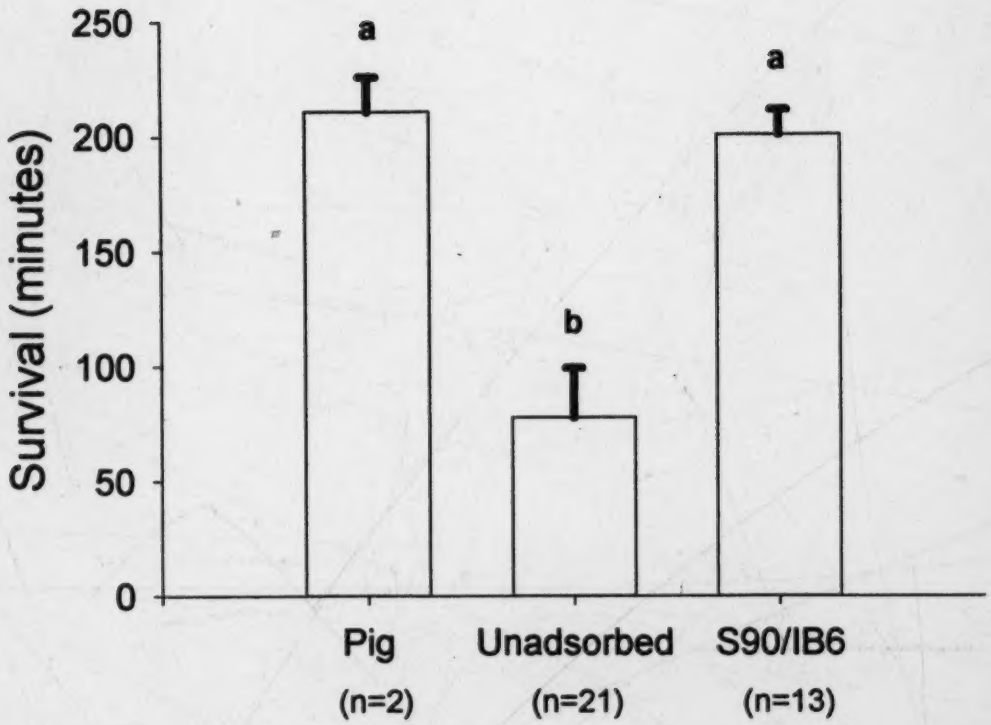

Figure 5.6 Survival of pig working hearts perfused with pig plasma, unadsorbed, or S90/IB6 adsorbed human plasma. There is a statistically significant difference ($p<0.001$, ANOVA) between the groups. Post-hoc Tukey testing showed S90/IB6 to be similar to pig and different from unadsorbed human plasma. Hearts perfused with S90/IB6 adsorbed plasma survive just as long as hearts perfused with pig plasma. The symbols **a** and **b** correspond to statistically different groups. Data are expressed as mean ± SEM.

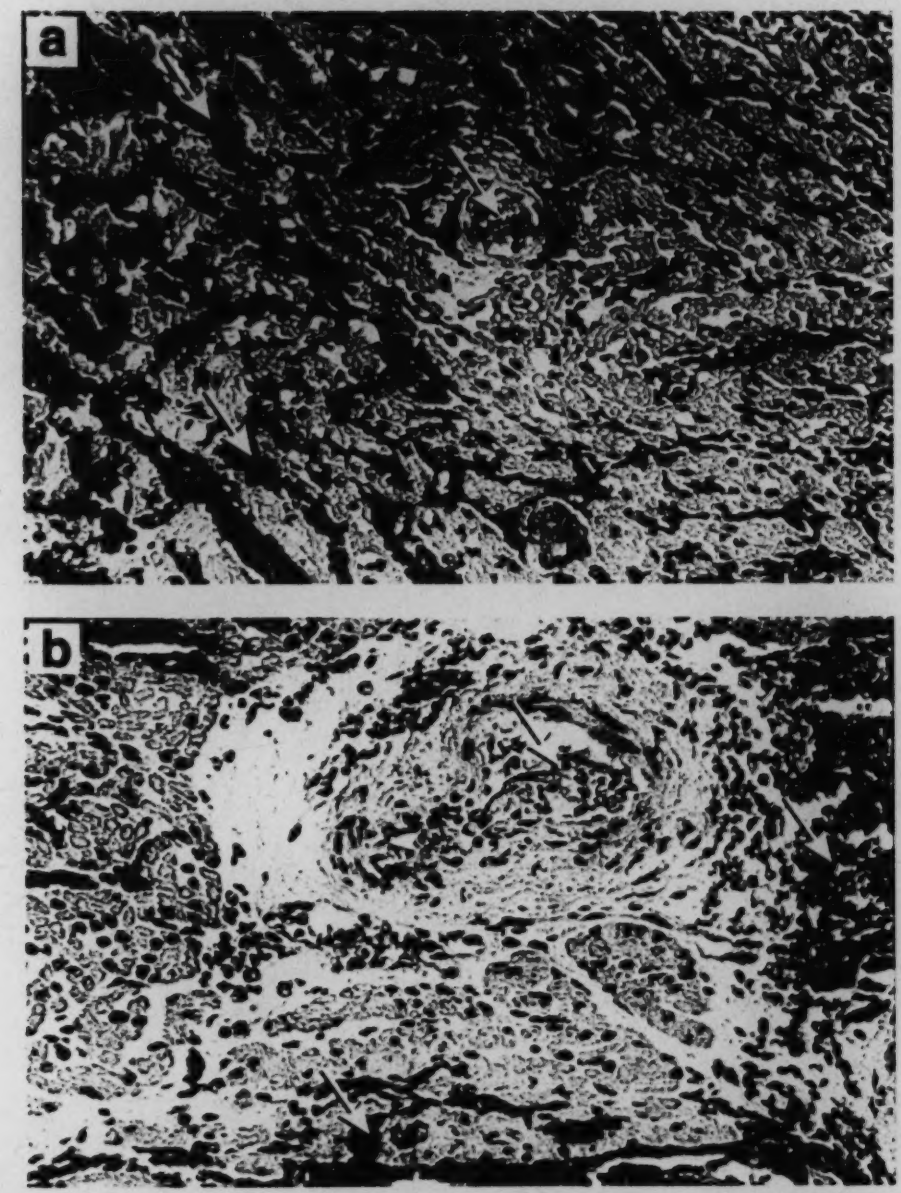

Figure 5.7. Hematoxylin and Eosin staining of pig working hearts perfused with a) blank (CP) adsorbed human blood or b) S90/IB6 adsorbed human blood. Both groups show evidence of hemorrhage (white arrows) and thrombosis (yellow arrows) consistent with hyperacute rejection. Magnification = 25x."

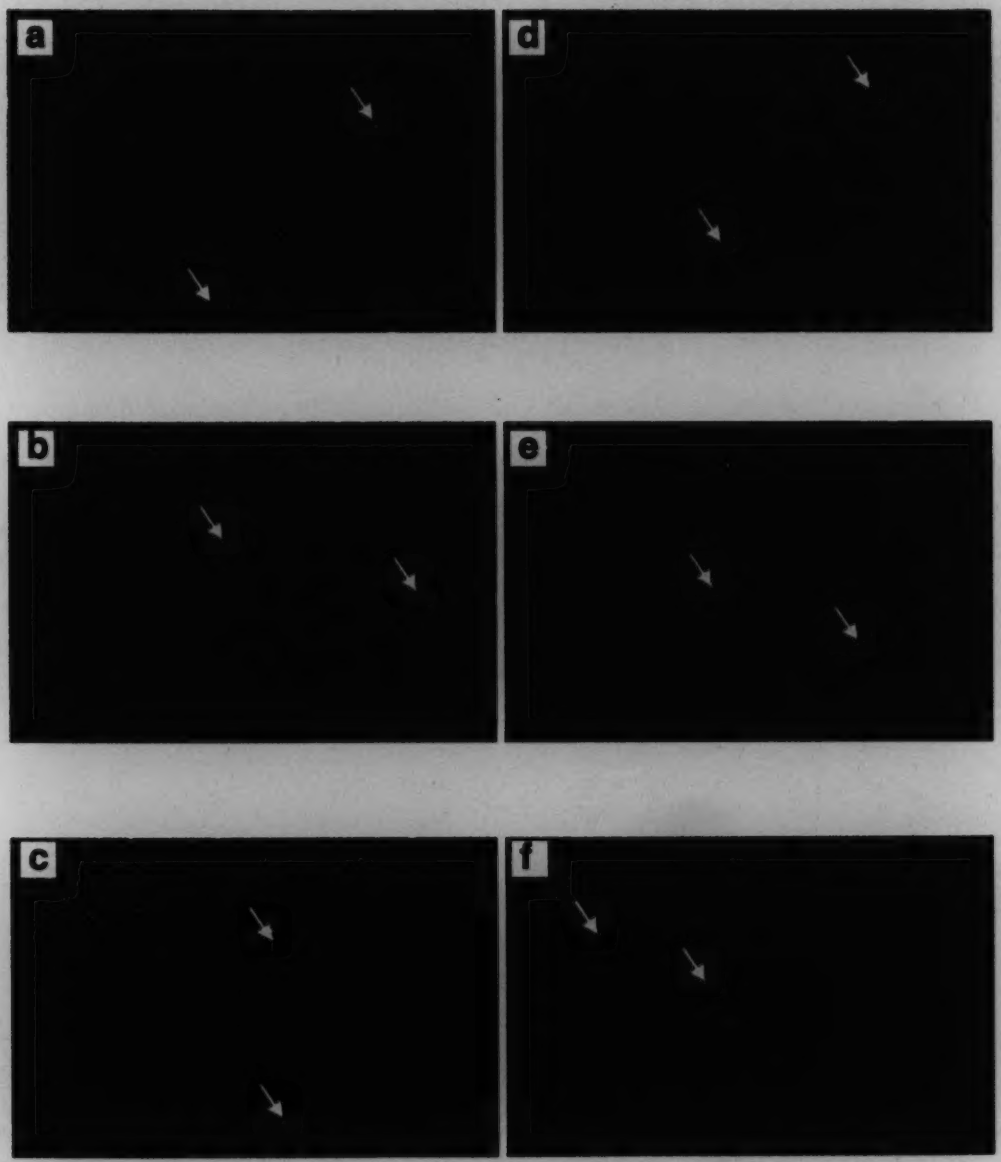

Figure 5.8. Immunofluorescence staining for human antibody and complement binding on pig working hearts. Hearts perfused with blank (CP) adsorbed human blood: a) human IgM, b) human IgG, c) C1q complement protein. Hearts perfused with S90/IB6 adsorbed human blood: d) human IgM, e) human IgG, f) C1q complement protein. Both groups show evidence of binding all three factors (arrows). Magnification = 100x.

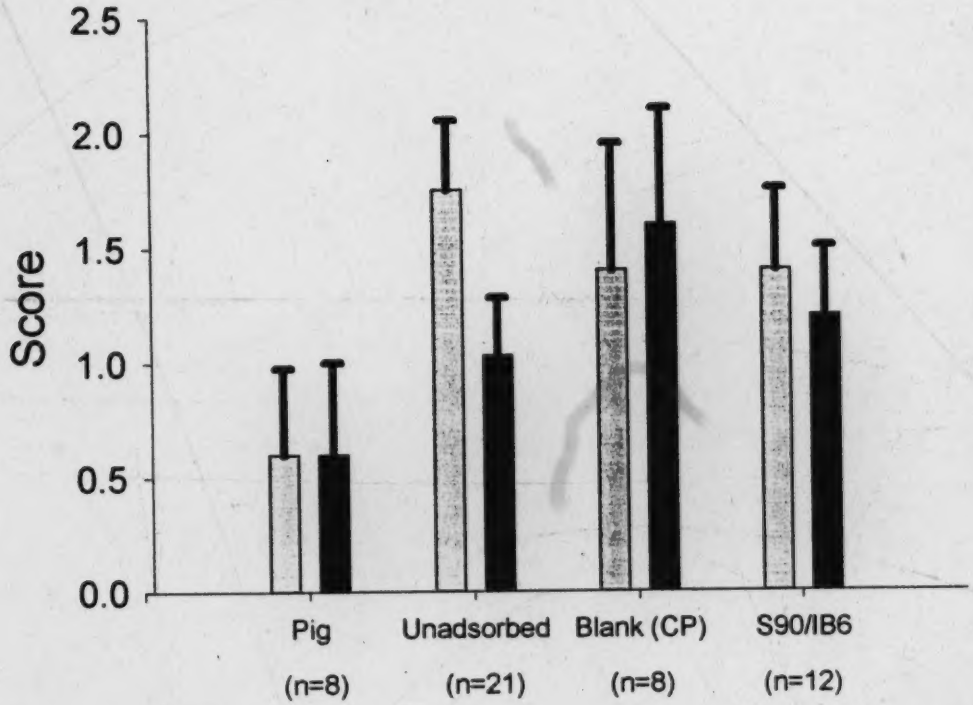

Figure 5.9 Light microscopy of pig working hearts perfused with pig whole blood, unadsorbed, blank (CP) adsorbed, or S90/IB6 adsorbed human whole blood. Sections were stained with hematoxylin and eosin and scored for evidence of hyperacute rejection (hemorrhage (□) and thrombosis (■)). There is evidence of hyperacute rejection in the unadsorbed, blank (CP) adsorbed, and S90/IB6 adsorbed groups. Data are expressed as mean ± SEM.

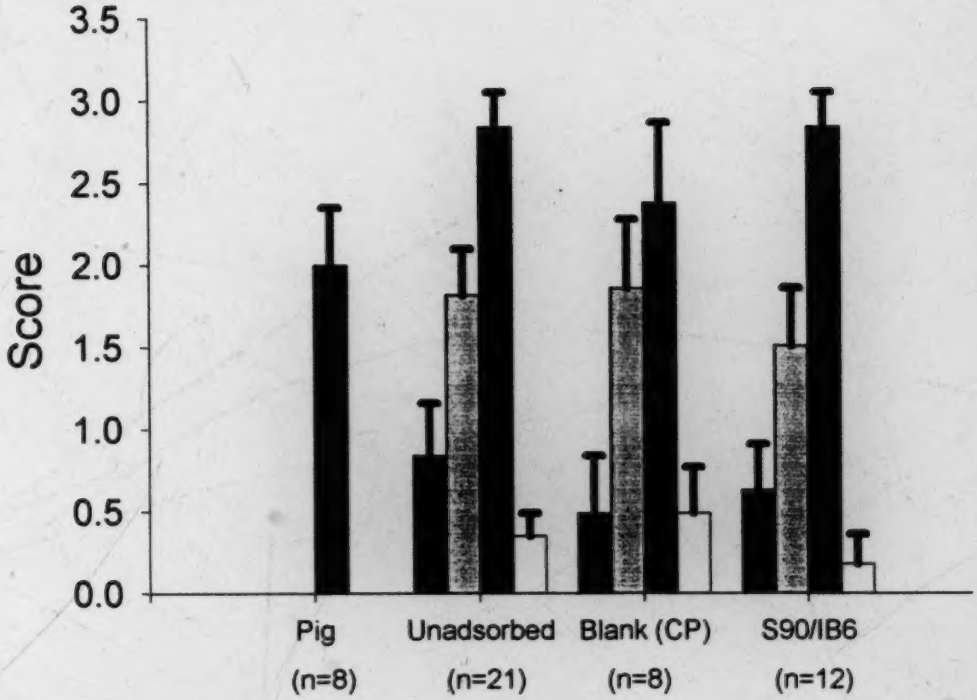

Figure 5.10 Immunofluorescence of pig working hearts perfused with pig whole blood or unadsorbed, blank adsorbed, or S90/IB6 adsorbed human whole blood. Sections were scored for binding of IgG (■), IgM (□), C3 (■), and C1q (□). There is antibody and complement binding on the unadsorbed, blank (CP) adsorbed, and S90/IB6 adsorbed groups. Data expressed as mean ± SEM.

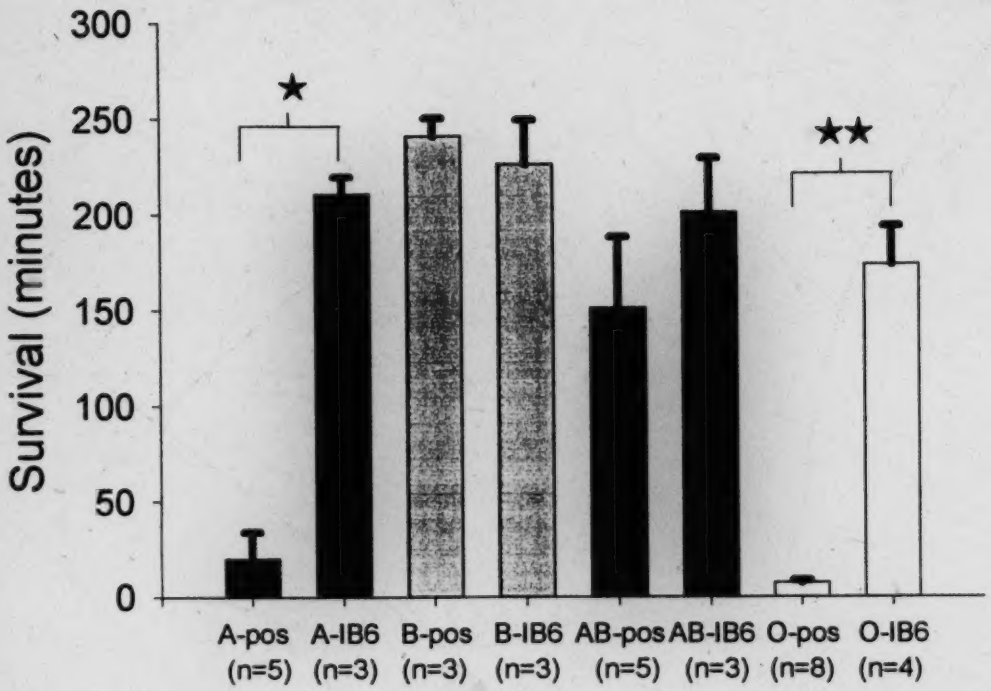

Figure 5.11 Survival of pig working hearts perfused with 15% unadsorbed (pos) or S90/IB6 adsorbed (IB6) human blood type A, B, AB, and O plasma. S90/IB6 adsorption statistically significantly improves survival compared to positive controls only in blood groups A and O (★: $p<0.001$, t-test and ★★: $p=0.003$, t-test). Data expressed as mean ± SEM.

Table 5.3 Cardiac function, creatine kinase change, and weight change for pig working hearts perfused with 15% unadsorbed or S90/IB6 adsorbed plasma from each blood group.

Group	Average 180 minute function (n) ((ml/min x mmHg x bpm)/1000)	Creatine Kinase Change (n) (U/L/min)	Weight Change (n) (g/min)
A-unadsorbed	40 ± 10 (5)	191 ± 112 (3)	3.3 ± 0.8 (5)
A-S90/IB6	2005 ± 183 (3)	17 ± 3 (3)	0.10 ± 0.01 (3)
	P=0.008 (t-test)	*P=0.160 (M-W)*	*P=0.036 (M-W)*
B-unadsorbed	1064 ± 360 (3)	12 ± 4 (3)	0.06 ± 0.01 (3)
B-S90/IB6	1936 + 62 (3)	13 ± 3 (3)	0.09 ± 0.01 (3)
	P=0.132 (t-test)	*P=1.00 (M-W)*	*P=0.200 (M-W)*
AB-unadsorbed	391 ± 149 (5)	74 ± 48 (5)	0.30 ± 0.18 (5)
AB-S90/IB6	2002 ± 134 (3)	26 ± 3 (3)	0.11 ± 0.01 (3)
	P<0.001(t-test)	*P=1.00 (M-W)*	*P=0.786 (M-W)*
O-unadsorbed	27 ± 9 (8)	365 ± 106 (7)	3.08 ± 0.421 (8)
O-S90/IB6	1630 ± 296 (4)	19 ± 3(3)	0.12 ± 0.02 (3)
	P=0.012 (t-test)	*P=0.017 (M-W)*	*P=0.012 (M-W)*

M-W=Mann Whitney U test (see text for details).
Data are expressed as mean ± SEM.

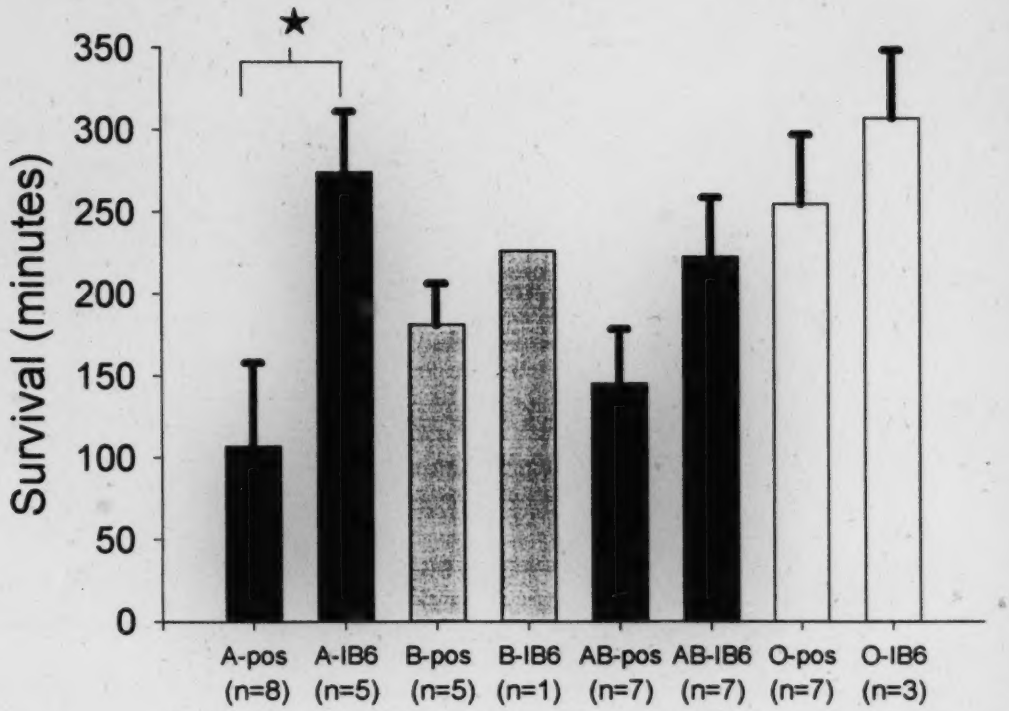

Figure 5.12 Survival of pig working hearts perfused with unadsorbed (pos) or S90/IB6 adsorbed (IB6) human blood type A, B, AB, and O whole blood. S90/IB6 adsorption statistically significantly improves survival compared to positive controls only in blood group A (★: p=0.039, t-test). Data expressed as mean ± SEM.

Table 5.4 Cardiac function, creatine kinase change, and weight change for pig working hearts perfused with unadsorbed or S90/IB6 adsorbed human whole blood from each blood group.

Group	Average 180 minute function (n) ((ml/min x mmHg x bpm)/1000)	Creatine Kinase Change (n) (U/L/min)	Weight Change (n) (g/min)
A-unadsorbed	832 ± 398 (8)	174 ± 81 (7)	3.68 ± 2.77 (8)
A-S90/IB6	1705 ± 298 (5)	24 ± 4 (5)	0.05 ± 0.01 (5)
	P=0.147 (t-test)	*P=0.07 (M-W)*	*P=0.06 (M-W)*
B-unadsorbed	2556 ± 1074 (5)	26 ± 15 (3)	0.08 ± 0.01 (5)
B-S90/IB6	786 (1)	19 (1)	0.06 (1)
AB-unadsorbed	1515 ± 339 (7)	258 ± 217 (6)	1.5 ± 1.4 (7)
AB-S90/IB6	3490 ± 933 (4)	28 ± 9 (4)	0.06 ± 0.01 (4)
	P=0.04 (t-test)	*P=0.35 (M-W)*	*P=0.41 (M-W)*
O-unadsorbed	2144 ± 380 (7)	30 ± 4 (7)	0.09 ± 0.02 (7)
O-S90/IB6	947 ± 148 (3)	16 ± 10 (2)	0.04 ± 0.004 (3)
	P=0.08 (t-test)	*P=0.33 (M-W)*	*P=0.04 (M-W)*

M-W=Mann Whitney U test. See text for details.
Data are expressed as mean ± SEM.

Table 5.5 Survival, cardiac function, creatine kinase change and weight change of pig working hearts perfused with unadsorbed (O-red blood cell, any blood group plasma) or S90/IB6 adsorbed (regardless of red cell or plasma blood type) human whole blood.

Group	Survival (n) (minutes)	Average 180 MinuteCardiac Function (n) ((ml/min x mmHg x bpm)/1000)	Creatine Kinase Change (n) (U/L/min)	Weight Change (n) (g/min)
Unadsorbed (with O red blood cells)	226 ± 25 (17)	1985 ± 356 (17)	40 ± 12 (16)	0.14 ± 0.07 (17)
S-90/IB6 (regardless of red cell type or blood group)	262 ± 21 (13)	2009 ± 412 (13)	23 ± 4 (12)	0.05 ± 0.07 (13)
P-value	*0.296 (t-test)*	*0.966 (t-test)*	*0.423 (M-W)*	*0.09 (M-W)*

M-W=Mann Whitney U test
Data are expressed as mean ± SEM

Table 5.6 Known non-α-gal carbohydrate antigens against which humans can have naturally occurring antibodies (15,37,38).

1. A: GalNAcα 1-3(Fucα 1-2)Galβ1-4GlcNAcβ-R[(a)]
2. B: Galα 1-3(Fucα 1-2)Galβ1-4GlcNAcβ-R
3. Thomsen-Friedenreich (T or TF) Galβ1-3GalNAcα 1-R
4. Tn (TF precursor) GalNAcα-R
5. Sialosyl-Tn: NeuAcα 1-R
6. p^K: Galα 1-4Galβ1-4Glcβ1-R
7. Other P antigens
8. Sulfatide I: SO_4-3Gal-R
9. Forssman: GalNAcα 1-3GalNAcβ1-3Galα 1-4Galβ1-4Glcβ1-R
10. i: Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-R[(b)]
11. I: Galβ1-4GlcNAcβ1-3(Galβ1-4Glcβ1-6)Galβ1-4GlcNAcβ-R[(b)]
12. α Rhamnose-containing oligosaccharides
    L-Rhm-α-Rhm
    L-Rhm-α 1-3GlcNAcβ1-2L-Rhm-α-R
13. βGlcNAc-containing oligosaccharides
    GlcNAcβ-R
    GlcNAcβ1-4GlcNAcβ-R

(a) R are glycolipid or glycoprotein carrier molecules anchored in the cell membrane.
(b) The core structures of the ABH antigen system which are fucosylated by H transferase to generate H substance.

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CHAPTER 6

Conclusions and Future Directions

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There is a critical organ shortage crisis present today in allotransplantation and the crisis is only expected to get worse if things stay the way they are now (1,2). Many methods to increase the supply of organs are being pursued (3) – xenotransplantation is one of the more promising ones.

The pig appears to be the most promising organ donor for various anatomical, physiological, practical, and economical reasons (4). Though there are potential concerns with the use of pig organs (such as infection transmission) (5,6), xenotransplantation has the potential to save many lives. Some of the specific advantages of xenotransplantation include an endless supply of organs and the ability to genetically modify tissues and organs to potentially make pig organs better than human organs for transplantation (7). However, there are barriers to successful porcine-human organ xenotransplantation. The first major barrier is hyperacute rejection generally caused by anti-α-gal antibodies (8). There are many questions related to hyperacute rejection which have not been fully answered. This project looked at important issues related to hyperacute rejection of porcine hearts by human blood.

The project completed has added very important new knowledge to the area of porcine-to-human xenotransplantation. Using a validated model of hyperacute rejection, it was determined that survival time is dependent on the anti-α-gal antibody level and the type of human red blood cells perfusing the heart, but not other variables such as the anti-pig antibody levels. This is important clinically as pre-transplantation, one just needs to measure anti-α-gal antibody levels which is an easier and less expensive assay to perform

rather than anti-pig aortic endothelial cell assays. Also, transfusion of O red blood cells (the universal blood donor) to patients may be a useful part of the protocol to try to help prolong graft survival.

This project also showed the novel finding that there is a human ABO blood group difference in porcine-to-human xenotransplantation. People who are blood group A are least likely to accept a xenograft without prior immunoadsorption and blood group B are more likely to accept a xenograft (probably with very minimal immunoadsorption). The mechanism involved in giving the blood group differences appears to be that the anti-B antibody augments rejection, and the anti-A antibody may protect against rejection. Finding that there is a blood group difference also has important clinical implications. When human clinical trials are started, perhaps people most likely to succeed (blood group B) should be xenotransplanted with immunoadsorption, O red blood cell transfusion using transgenic pigs and immunosuppressive drugs. This will hopefully be the ideal combination and if it fails significantly, then the scientific community will need to re-think whether xenotransplantation is a viable option for the organ shortage crisis. In addition, there are certain ethnicity's (in eastern Asia) that have a much larger proportion of blood group B people in their population (9). Many eastern Asian countries do not routinely harvest organs from brain dead individuals for cultural reasons and thus this adds to organ shortages (10). These people may benefit the most from xenotransplantation.

This project also demonstrated the novel finding of the usefulness of the S90/IB6 immunoadsorbent (and anti-α-gal/ anti-α-gal like depletion) in improving survival of pig hearts perfused with human blood and the fact that the amount of benefit depends on the

human ABO blood group and the presence of red blood cells (especially O red blood cells). These findings also have important clinical implications. Immunoadsorption has its problems with cost and practicality being two of them. Knowing who would benefit from immunoadsorption and who would not (or who would need less immunoadsorption) would be useful information to clinicians.

There is much more work to do in the future. Extending the survival of the porcine working hearts by perhaps attaching a dialysis filter to the circuit may help the hearts to survive much longer enabling one to study later events in xenograft rejection as well as allowing better detection of differences between groups. For example, if the negative control (autologous pig blood) hearts could function for 12 hours, one may see large statistically significant differences in survival between the negative controls and the S90/IB6 group which would strengthen the importance of addressing the other antibodies (besides anti-α-gal) that are still present. Also by extending survival, one may see a survival benefit for blood group B plasma after S90/IB6 treatment which would help clarify whether S90/IB6 is or is not useful in that blood group. As humans are likely going to be transplanted with adult pig organs, one could build a bigger perfusion system and repeat some of the experiments in small numbers to ensure findings are the same as when piglet hearts were used. Similar findings would be expected and one would only want to do a few experiments as there may be practicality issues with obtaining enough human blood to be able to run a large perfusion circuit.

Other important studies for the future include studying human red blood cells, studying the anti-A and anti-B antibodies and the S90/IB6 immunoadsorbent. Determining why human O red blood cells have such a protective effect would be

important to know to be able to use that knowledge for clinical xenotransplantation. Doing studies on the actions and interactions of the anti-A, anti-B and anti-α-gal antibodies would also be important. Studying the S90/IB6 immunoadsorbent in more detail such as determining if it depletes cytokines or other factors and refining the immunoadsorbent to make it more practical and less expensive would be important. Determining the specific identity of the antibodies that still bind to the pig heart after S90/IB6 immunoadsorption and the importance of these antibodies would also be important for future clinical xenotransplantation. Thus, there is still much work to be done but much has been accomplished.

In the early 1960's when heart surgeons were having dismal success rates with heart transplantation, they persevered and with future research, we have now had successful heart transplants performed for over 20 years. Xenotransplantation has very great potential but like all other major advancements, it has had, and will likely continue to have periods of set backs and confusion. Perseverance and continued research (to which this project has contributed) should prove xenotransplantation to be successful in the near future.

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